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Isolations and Some Properties of Native Lipoproteins from Egg Yolk*

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(Received for publication, April 19, 1961)

It has been inferred that egg yolk lipids are not bound to protein because about a half of the total lipid can be extracted by ether. Most of the earlier studies dealt with the isolation and properties of yolk lipoproteins in a state that these "free" lipids had been removed by ether extraction (*1*). However, recently it has become evident by ultracentrifugal studies (*2–5*) that nearly all egg yolk lipids are bound to protein. Thus it is considered to be important to obtain the informations on the native lipoproteins prepared by a method not involving ether extraction.

This paper describes the method of isolation of two groups of native lipoproteins from egg yolk and some informations on their properties. One group is the low density lipoprotein (LDL) comprising about 84% lipid, the other the high density one (HDL) with about 20% lipid.

EXPERIMENTAL METHODS

Materials—The yolks of the fresh eggs of White Leghorn were washed in tap water and rolled on filter paper to free them from adhering white. The yolk membranes were then punctured and the contents allowed to drain out.

Analytical Methods—Nitrogen was determined by micro Kjeldahl or Dumas method, phosphorus by the method of Allen (*6*), cholesterol by the method of Sperry (*7*), and total lipids as described previously (*8*). These values were all estimated on dry basis.

Physical Methods—Sedimentation experiments were performed in a Spinco Model E ultracentrifuge. For ultracentrifugal isolation of LDL was used a Spinco Model E or L instrument. Electrophoresis was carried out in a Tiselius apparatus with a Schlieren-diagonal optical system, and also by the agar gel method according to Giri (*9*). For staining protein Amidoschwarz 10 B was used and Sudan Black for lipids. The pH was measured with Beckman Model G instrument at room temperature. Viscosity measurements were made at 20°C in an Ostwald Viscosimeter. Solubility curves were obtained by measuring the optical transmittancy at 650 mμ with a Beckman DU spectrophotometer.

Isolations of HDL and LDL by Dilution Method—To yolk contents were added a fifth volume of 0.6 *M* magnesium sulfate solution and the mixture was stirred thoroughly and allowed to stand for half an hour at room temperature. Water was then added slowly until the concentration of magnesium sulfate became 0.02 *M*. After a few hours the produced precipitate was collected by centrifugation at 4,000 r.p.m. for 40 minutes. The supernatant was decanted. The precipitate was dissolved in 1.2 *M* magnesium sulfate solution to make a volume a fourth of the original yolks. After an hour the solution was diluted to 0.03 *M* by adding water slowly. After standing for several hours the produced precipitate was centrifuged, and dissolved again in 1.2 *M* magnesium sulfate. The solution was diluted to 0.09 *M*. After standing overnight the yellow sticky precipitate was removed by centrifugation and the supernatant was diluted further to 0.03 *M*. The produced precipitate of HDL was collected by centrifugation after standing for several hours. In some cases the precipitate was recentrifuged after suspending in water, since the solubility of HDL was reduced by the magnesium sulfate adhered to the precipitate. On the other hand, the first supernatant from 0.02 *M* magnesium sulfate was further diluted to 0.001 to 0.0005 *M* and allowed to stand overnight in a cold room. The floated yellow LDL was separated from the underlayer by siphoning and then collected by centrifuging. The yields of the HDL and LDL from 30 g. of yolks were about 1.0 and 4.5 g. in dry weight, respectively.

The HDL and LDL prepared by the dilution method contained small amounts of floating and sedimenting components, respectively. These could not be removed by reprecipitations, but the ultracentrifuge was effective to remove them.

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* The gist of this work was presented at the 10th Annual Meeting of the Society of Electrophoresis of Japan, September 26, 1959.

Isolation of LDL by Ultracentrifugal Method—The yolk solution which was added with 0.6 M magnesium sulfate as described above, was diluted to 0.03 M and then centrifuged. Solid sodium chloride was added to the supernatant to about 10 per cent. Centrifuge tubes were four fifth-filled with this solution and water or buffer solution was layered on top. After centrifugation for 3 hours at 40,000 r.p.m. the contents of the tubes were distributed into four distinct zones: a yellow semisolid layer on the surface (A), a yellow condensed layer occupying the top third of the tube (B), a clear and almost colorless liquid region (C), and precipitate (D). Since the A and B zones represented a similar lipid content, both were combined, but if necessary, were collected separately. The yield of the LDL by this method was far better than by the dilution method. As resolved in the ultracentrifuge there was no sedimenting components in the semisolid layer A, whereas in the condensed layer B a very small amount of sediment was recognized, but it could be removed by recentrifugation after diluted again with 10% NaCl.

RESULTS

Ultracentrifugation of Whole Egg Yolk—Fig. 1A represents the result of ultracentrifugation of whole egg yolk diluted with carbonate buffer, pH 9.8 and μ 0.15. Three sedimenting ($S\eta_{20,w}$*=9.5S, 6.5S, and 3.5S) and two floating ($S\eta_{20,w}=-20$ S and -9 S) components could always be found. However, when egg yolk was diluted with 0.5 M NaCl, the 6.5S component almost vanished and, on the contrary, the 9.5 S peak became large (Fig. 1B). After removing the livetin fraction, a water-soluble part of yolk proteins, by water dialysis, a considerable decrease of the 3.5 S and a little lowering of the 6.5 S were observed (Fig. 1C). This change in the diagram was explained satisfactorily with the aid of separate experiment, which indicated that among three components of the livetin fraction α- and β-livetins are contained in the 3.5 S peak and γ-livetin in the 6.5 S peak.**

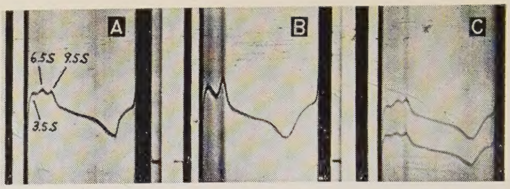

FIG. 1. Sedimentation diagrams of whole egg yolk solution in : A, carbonate buffer pH 9.8, μ 0.15; B, 0.5 M NaCl: C(upper), carbonate buffer pH 9.8, μ 0.15, after removing the livetin fraction by water dialysis; C(lower), same to A. The diagrams were taken 26(A), 22(B) and 27(C) minutes after rotor reached full speed (52,640 r.p.m.)

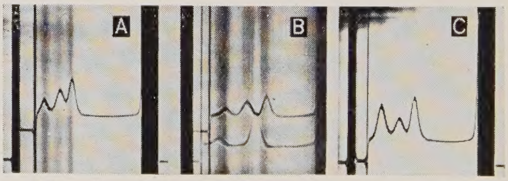

FIG. 2. Sedimentation diagrams of 0.03 M precipitate (A and B) and 0.09 M precipitate(C) in : A, carbonate buffer pH 9.8, μ 0.15; B(upper), same to A; B(lower), 0.5 M NaCl; C, carbonate buffer pH 9.8, μ 0.15. The diagrams were taken 36(A), 57(B) and 57(C) minutes after rotor reached full speed (50,740 (A), 56,100 (B), and 50,740 (C) r.p.m.).

Ultracentrifugation of Isolating Fractions—Fig. 2A shows a sedimentation diagram of the 0.03 M precipitate obtained at the first dilution step of whole egg yolk. It was verified by another experiment that the slowest peak (3 S) corresponds to phosvitin and the other two (9.5 S and 6.5 S) to HDL. In an early stage of sedimentation, a tolerable amount of floating fraction was furthermore observed. A similar diagram appeared in the precipitate obtained by diluting to 0.02 M. When a solvent of 0.5 M NaCl was used instead of the buffer, the 6.5 S component completely disappeared and the 9.5 S increased as much as the portion of vanished 6.5 S, whereas the 3 S peak persisted with the unchanged proportion (Fig. 2B). This implies that the 6.5 S component associated with each other in 0.5 M NaCl and phosvitin did not participate in this association. In the 0.09 M precipitate obtained from the 0.03 M fraction, phosvitin was concentrated remarkably as compared with the other two, especially with the 6.5 S component (Fig. 2C).

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* $S\eta_{20,w}$ is the sedimentation constant at 20°C corrected for the viscosity alone.

** The proportion of α plus β-livetins to γ-livetin was $\alpha+\beta:\gamma=5:2$ (*10*).

A typical sedimentation diagram of HDL fraction obtained from the 0.09 M supernatant is shown in Fig. 3A. This fraction generally consisted of nearly equal amount of 9.5 S and 6.5 S components. However, the HDL having different compositions could be prepared by the stepwise dilution between 0.09 M and 0.03 M, namely, the fraction precipitating at the first stage of dilution was rich in 9.5 S component (Fig. 3B). This will provide, accordingly, a possibility for the separation of 9.5 S and 6.5 S components by the repeated stepwise dilution. Actually, after nine times reprecipitations at 0.07 M, the obtained material consisted of almost pure 9.5 S component (Fig. 3C, upper). On the other hand, it was found that the ratio of 9.5 S and 6.5 S in the HDL fraction remarkably depends on the pH and ionic strength of the solvent. Generally an increase of ionic strength was followed by a reduction of 6.5 S and an increase of 9.5 S component. This effect was observed more remarkably in the solvent of neutral pH. At pH 6.0, 6.8, and 7.8 the 6.5 S component disappeared perfectly by an addition of NaCl to 0.5 M and only 9.5 S component was evident (Fig. 3D), whereas at pH 8.6 and 9.8, only a little decrease of 6.5 S component was shown (Fig. 3E). At pH 9.8, an addition of NaCl to a concentration over 1.5 M was necessary to vanish the 6.5 S almost completely*. Based on the result of the molecular weight estimations (see the latter section), the converison of 6.5 S to 9.5 S are considered to correspond to the dimerization of 6.5 S component. It was furthermore confirmed that the produced dimer dissociated again into the 6.5 S unit by dialysing against the buffer of pH 9.8 and μ 0.15.

From the above results it could be inferred that there exist two components, H_1 and H_2, in the HDL fraction; H_1 is stable in the buffer of pH 9.8 and μ 0.15, indicating an almost single peak (9.5 S) as shown in Fig. 3C (upper); the other, H_2, a major part of which dissociates into the half (6.5 S) at pH 9.8 and μ 0.15 and again dimerizes by addition of NaCl or lowering of pH. However, as shown in Fig. 3C (lower), in a buffer of pH 11.0 nearly half of H_1 dissociated into the 6.5 S unit. Therefore, it was concluded that the HDL fraction consists of two components with the different intermolecular affinity.

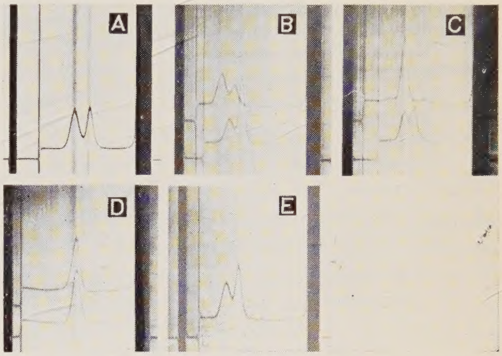

Fig. 3. Sedimentation diagrams of HDL(A), 6.5 S rich (B, upper) and 9.5 S rich (B, lower) fractions obtained by the stepwise dilution of HDL, H_1 (C) obtained by the nine times reprecipitations at 0.07 M, and HDL(D and E) in : A, B, and C(upper), carbonate pH 9.8, μ 0.15; C(lower), carbonate pH 11.0, μ0.15; D(upper), phosphate pH 7.8, μ 0.1 plus 0.5 M NaCl; D(lower), phosphate pH 6.8, μ 0.1 plus 0.5 M NaCl; E, carbonate pH 9.8, μ 0.15 plus 0.5 M NaCl. The diagrams were taken 44(A), 40(B), 46(C), 62(D), and 46(E) minutes after rotor reached full speed (50,740 r.p.m.).

Fig. 4A represents a diagram of LDL fraction prepared by the dilution method. Besides two major components (L_1 with −20 S and L_2 with −9 S), there existed a small amount of sedimenting species which could be removed completely neither by repeated dilution nor by dilution to a concentration under 0.02 M, but easily removed by means of ultracentrifugation. As evident from the diagrams (Figs. 1, 4A and 4B), a larger quantity of L_1 was contained in the LDL fraction than in the whole egg yolk or the 0.02 M supernatant. This fact implies that L_1 precipitated prior to L_2 when the magnesium sulfate concentration was lowered. Accordingly,

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* $(NH_4)_2SO_4$ and $MgSO_4$ were far more effective than NaCl in the order mentioned.

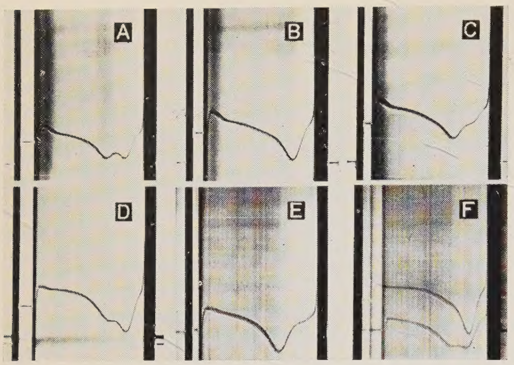

FIG. 4. Sedimentation diagrams of LDL(A) isolated by the dilution method, 0.02 *M* supernatant(B), L_1 rich fraction(C) precipitating between 0.025 *M* and 0.002 *M*, L_2 rich fraction(D) precipitating between 0.002 *M* and 0.0005 *M*, L_1 (E) prepared by three times reprecipitations at 0.001 *M*, and the top floating layer (F, lower) and the underlying condensed layer (F, upper) obtained by the ultracentrifugal method, all in carbonate pH 9.8, μ 0.15. The diagrams were taken 17(A), 19(B), 14(C and D), 22(E), and 19(F) minutes after rotor reached full speed (50,740 (all except B) and 52,640(B) r.p.m.).

as was the case for the HDL, this provides a possibility for the separation of L_1 and L_2. Figs. 4C and 4D indicate the result of the stepwise dilution between 0.025*M* and 0.0005*M*. Furthermore, after three times reprecipitations at 0.001 *M* the obtained material was 80 to 90% pure relating to L_1, as shown in Fig. 4E.* Unlike to the HDL fraction, the proportion of L_1 and L_2 was constant regardless of the pH of the solvent or the addition of NaCl. Fig. 4F represents a comparison of the two top layers, A and B, collected separately after ultracentrifugation. Although a considerable difference exists between the diagrams of both fractions, their lipid contents were similar with each other (about 84 per cent). The A layer comprised nearly equal amount of L_1 and L_2, resembling with the LDL prepared by the dilution method, but no sedimenting peak was observed at all. Thus it is obvious that the dilution method gives a low yield of the LDL owing to the loss of considerable amount of L_2, in spite of its superiority in the point of simplicity for preparing the LDL. Further studies for the complete separation of L_1 and L_2 are now in progress.

Physical Measurements—For two peaks of the HDL fraction, the corrected sedimentation constants ($S_{20,w}$) of 9.51 and 6.52 were calculated, respectively. Viscosity measurement presented a intrinsic viscosity $[\eta]$ of 0.054, and then, assuming the hydration of 0.3,* the frictional ratio of 1.25 was obtained. Therefore both components in the HDL, regardless to their dissociated or associated states, are considered to be rather close to spherical form (axial ratio under 3.0). Using a specific volume of 0.790 obtained by pycnometer measurement, the molecular weights of about 290,000 and 160,000 were estimated for 9.5 S and 6.5 S components, respectively. On the other hand, using $S_{20,w}=9.94$ and $[\eta]=0.045$ obtained in 0.5 *M* NaCl, the molecular weight of 300,000 was obtained. This result suggests that the change from 6.5 S to 9.5 S by addition of salt or lowering of pH is based on the dimerization of 6.5 S component.

The floatation rate (S_f) of the LDL fraction was determined in media of various densities and corrected in a state of water at 20°C only relating to the viscosity. The S_f values of L_1 and L_2 in a solvent having a density of 1.063 (NaCl) were 51 S_f and 29 S_f, respectively. Estimations of the hydrated densities of L_1 and L_2 were made from the extrapolated values of the solvent densities (ρ) at which zero floatation velocity would occur on the S_f *vs.* ρ plots, Since it was not known whether S_f was dependent on concentration, the S_f *vs.* ρ measurements were made with lipoprotein concentration kept constant. The obtained S_f *vs.* ρ plots represented a straight line. Thus, for L_1 and L_2, the hydrated densities of 0.969 and 0.980 were estimated, respectively. From these results and $[\eta]=0.038$, it was concluded that both

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* The separation of L_1 and L_2 was also possible by the ammonium sulfate fractionation, in which at about 41% saturation the L_1 rich fraction began to precipitate, but this method seemed to be inferior to the method described in the text owing to accompanying a slight increase of the sedimenting component.

* The higher than 0.3 assumed to the hydration, the closer the molecular shape to the sphere.

L_1 and L_2 are almost spherical particles having the hydrated diameters of 310 and 250 Å, and the hydrated molecular weights of 9,000,000 and 4,800,000, respectively.*

Electrophoresis—Electrophoresis of the HDL fraction by means of Tiselius apparatus indicated that this fraction consists of two electrophoretic components, having the descending mobilities of -6.2 and -5.2 ($\times 10^{-5}$ cm^2. $volt^{-1}$. $second^{-1}$) at pH 9.8 and μ 0.15, respectively (Fig. 5). Agar gel electrophoresis also represented the existence of two components as indicated by protein and lipid stainings. On the other hand, boundary electrophoresis failed to obtain reliable results on the LDL fraction because it was difficult to maintain stable boundaries owing to its low density property, but the agar gel electrophoresis demonstrated the presence of only one moving zone as detected by both protein and lipid stainings, its electrophoretic velocity being close to that of the fast moving component of the HDL.

From a series of electrophoreses of the HDL fraction having various ultracentrifugal compositions, it was suggested that the fast and slow moving components in electrophoresis corresponded to the two components, H_1 and H_2, respectively, rather than the 9.5 S and 6.5 S components.

Chemical Analyses—The results of chemical analyses of the HDL and LDL fractions are summarized in Table I. Both fractions were prepared by the dilution method and consisted of nearly equal amount of two ultracentrifugal components (Figs. 3A and 4A). As to the lipid

Table I

Summary of Chemical Analyses

Material	HDL	LDL
	%	%
Nitrogen	12.1	2.75
Phosphorus	0.813	0.751
Lipids	20	84
Cholesterol[1]	5	4
Phospholipid[2]	53	24
Triglyceride	42	62
Protein	80	16
Nitrogen	14.6	14.7
Phosphorus	0.514	0.307

1) Predominantly free cholesterol.
2) Calculated as lecithin.

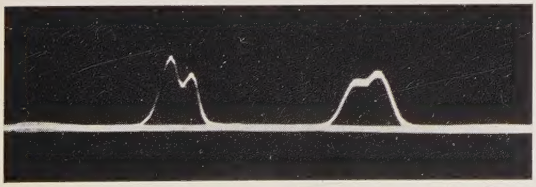

Fig. 5. Electrophoretic diagram of HDL fraction in carbonate pH 9.8, μ 0.15, after 200 minutes at a potential gradient of 2.85 volt./cm.

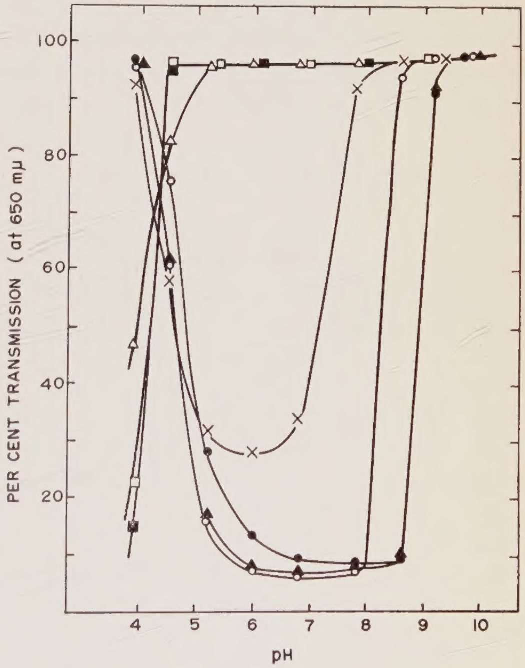

Fig. 6. Solubility of HDL at various pHs and ionic strengths; protein concentration, 0.1%: pH 3.9, 4.5, and 5.2 (acetate); pH 6.0, 6.8, and 7.8 (phosphate); pH 8.6 (veronal); pH 9.2 and 9.8 (carbonate): Increase of ionic strength depends on NaCl: ●, 0.05μ; ▲, 0.1μ; ○, 0.15μ; ×, 0.2μ; △, 0.3μ; □, 0.4μ; ■, 0.5μ.

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* The molecular weights were calculated according to the following relation (*11*): $S\eta_{20,w}\times 10^{13}=K(D-\rho)$, D and ρ are the densities of hydrated lipoprotein and solvent medium, respectively, K is directly proportional to the square of diameter, d^2, for spherical molecule, namely, $K=(d\times 10^8)^2/182$, $(d\times 10^8)$ is a diameter of hydrated molecule (in Ångstrom unit).

content, values similar to those in Table I were obtained with each of the HDL and LDL fractions having various ultracentrifugal compositions. It will be inferred from this that there exists hardly any difference in the lipid content between H_1 and H_2 or L_1 and L_2.

Solubility Properties—Solubility properties of both the HDL and LDL depended remarkably on the pH and ionic strength of the solvent. Fig. 6 depicts the solubility curves obtained by changing the ionic strength from 0.05 to 0.50 in the pH range between 3.9 and 10.0. Beyond pH 9.0 both the HDL and LDL represented very high solubilities throughout the ionic strength examined. In the neutral pH region the solubilities decreased with the lowering of ionic strength, so that the ionic strength over 0.3 was necessary to keep the solution clear. At the acid side below pH 4.3 the correlation between the solubility and ionic strength was reversed, especially remarkably in the case of HDL, namely, the solubility was reduced with the increase of ionic strength.

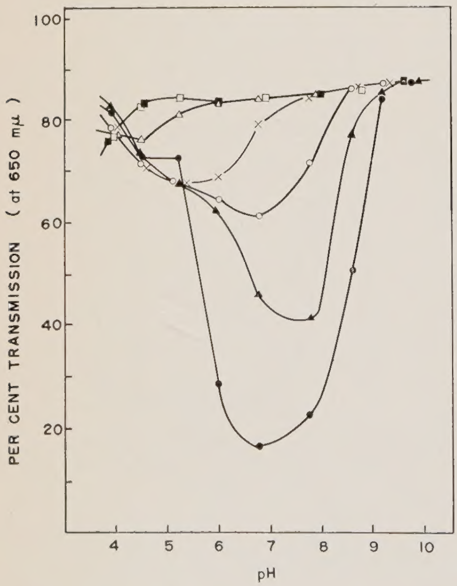

FIG. 7. Solubility of LDL at various pHs and ionic strengths; protein concentration, 0.2%: Conditions of pH and ionic strength are same as Fig. 6.

DISCUSSION

In the present work ultracentrifugal and electrophoretic analyses showed that egg yolk contains two groups of native lipoproteins, HDL and LDL, each of which consists of two species of lipoproteins. The proportion of these lipoproteins in total yolk proteins could be estimated roughly* from the sedimentation diagram of the diluted egg yolk. Thus the HDL and LDL formed 16 and 72 per cent of the total, respectively. The residual 12 per cent corresponded to phosvitin and livetins. On the other hand, the ratio of H_1 to H_2 in the HDL was close to unit, while that of L_1 to L_2 was 1 : 2. Therefore the final ultracentrifugal composition became as follows: L_1, 24; L_2, 48; H_1, 8; H_2, 8; livetins,** 7.5; phosvitin,** 4.5 per cents.

Both H_1 and H_2 are in equilibrium with their half unit in alkaline solution, but the dissociation of the former occurred at more alkaline pH than the latter. Accordingly, when the sedimentation diagram of HDL fraction was obtained at alkaline pH, the apparent composition estimated from the diagram would never provide the true ratio of H_1 to H_2. At neutral pH the HDL fraction showed a single sedimenting peak as a result of dimerization. The result obtained here suggested that hardly any phosvitin took part in this dimerization (Fig. 2B). Joubert and Cook (*12*) supposed a complex of three proteins—lipovitellin, phosvitin, and γ-livetin—for the major sedimenting component (10 S) of egg yolk, which yielded only one peak when examined in NaCl solution. However, if such a complex is formed, an appearance of sedimenting component having S value larger than 10 S should be expected. It is considered likely that the γ-livetin they observed was probably the dissociated product of HDL. Actually they observed that γ-livetin could be resolved from the complex in buffer of pH 9.0. In preliminary experiments with a mixture of HDL and isolated γ-livetin or γ-livetin alone any interaction or association was not observed to occur in neutral pH or in salt solution. Therefore, in the present work, no evidence for the presence of such a complex as supposed by Joubert and Cook was obtained.

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* No corrections were made for differences in the dn/dc of the components, for the Johnston-Ogston effect, or the dilution effect due to the sector shape of the cell.

** These values were calculated by using the ratio reported in previous paper (*13*) (livetins : phosvitin = 14.2 : 8.5).

A lipoglycoprotein, X_2, recently separated from the egg yolk granules by Schjeide and Urist (*5*), seemed to be equivalent to the HDL in this work, since both resembled with each other in respects to the lipid content, the sedimentation behaviors, and the polysaccharide reaction (*14*).

In previous work (*14*), the author isolated two major lipoproteins from egg yolk by the method involving the ether extraction, the lipoproteins being provisionally termed "α- and β-lipovitellins", respectively, to avoid the confusion which arose from the numerous terms employed in the literature with respect to egg yolk components. In accordance with the anticipation from the preparation method, α-lipovitellin coincided to the HDL after ether extraction which removed about two third of the lipids bound to the HDL. As reported in the previous paper (*15*), the sedimentation diagram of α-lipovitellin, which gave a very wide peak having a summit at 17 S to 20 S, suggested that secondary associations of the molecules were accompanied by removing the bound lipids. The solubility of HDL decreased evidently after the ether extraction. This is probably due to either partial denaturation of the protein moiety or the increase of hydrophobic surface on the molecule by the ether extraction, but these require further investigation.

Most methods for the fractionation of yolk proteins involved the use of ether for the extraction of yolk lipids inferred to be free. However, recent ultracentrifugal studies suggested that nearly all yolk lipids were bound to protein (*2–5*) and that the lipid-protein complex comprised 80 to 90% lipid (*4*, *16*, *17*) and represented 25 to 30 S_f in density 1.063 (*16*) or 7.5 $S^{\circ}_{f20,w}$ (*17*). Furthermore, the present work has indicated the presence of two species of LDL, one of which evidently corresponds to the component described by above workers. The rather rapid broadening of the sedimentation peaks, however, suggests that both species of lipoproteins consist of closely similar and related macromolecules. Such a relation of reversible association or dissociation as observed in the HDL could not be found between two species of LDL despite of the anticipation from their molecular weights and the similarity in the lipid contents.

SUMMARY

1. The isolation methods of two groups of native lipoproteins (having 84 and 20% lipids) from egg yolk were described.

2. Physical measurements, chemical analyses, and solubility examinations were performed on these preparations.

3. Ultracentrifugal and electrophoretic studies found that each lipoprotein fraction consists of two species of lipoproteins, respectively.

4. The proportions of four lipoproteins, phosvitin, and livetins in the egg yolk were roughly estimated on the basis of the ultracentrifugal analysis.

5. The native lipoproteins obtained here were discussed comparing with the components reported by the previous workers.

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The Journal of Biochemistry, Vol. 50, No. 6, 1961

Electron Transporting Components Participating in Nitrate and Oxygen Respirations from a Halotolerant *Micrococcus*

II. Properties of Cytochrome c^{551} and Brown Protein

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In a previous paper of this series, the methods for purification of five chromoproteins, that is, cytochrome c^{551}, brown protein, cytochrome b$_4$ (I) and b$_4$ (II) and HdR* (cytochrome 625, 553) were described (*1*). Characterization of cytochromes b$_4$(I) and b$_4$ (II) was also described there in detail. The purpose of this comunication is to describe the properties of cytochrome c^{551} and the brown protein. These chromoproteins were isolated from a halotolerant denitrifying *Micrococcus.* Cytochrome c^{551} resembled mammalian cytochrome c in absorption spectrum and oxidation-reduction potential which was found to be approximately +0.249 volt., but had a lower isoelectric point than the latter. Chemical nature and physiological function of the brown protein is obscure at present.

MATERIALS AND METHODS

Bacterial Culture—A halotolerant denitrifying *Micrococcus*, stain No. 203 was used. Cultivation of the bacteria,** extraction and purification of chromoproteins were performed, by methods described in the previous paper (*1*).

Chemical and Physical Determinations—Sedimentation studies were performed with Spinco model E ultracentrifuge. Electrophoretic mobilities for cytochrome c^{551} and the brown protein were determined with a Hitachi Tiselius type electropholesis apparatus using 0.05 *M* phosphate buffer, pH 7.0 at 4°C. The oxidation-reduction protentials were measured at 25°C at pH 7.0 using ferricyanide—ferrocyanide system according to the method of Davenport and Hill (*2*). Spectrophotometric observations were made by means of a Hitachi model EPU-2 Spectrophotometer using 1 cm. cells. Protein was determined by the method of Lowry *et al.* (*3*).

RESULTS

Spectroscopic Properties of Cytochrome c^{551} and the Brown Protein—Cytochrome c^{551} and the brown protein were purified by the column chromatography on DEAE-cellulose, which

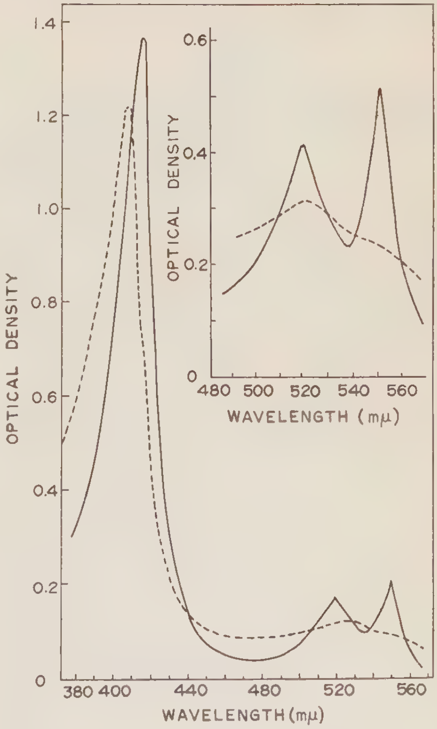

Fig. 1. Absorption spectra of cytochrome c^{551}. ——: Reduced form (with $Na_2S_2O_4$), ⋯: Oxidized form.

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* Abbreviations: HdR, hydroxylamine reductase; DEAE-cellulose, N,N'-diethylaminoethyl-cellurose.

** The large scale cultivation of the bacteria was carried out by courtesy of Nagoya Factory of Fujisawa Chemicals Co. Ltd.

was previously equilibrated with 0.1 M ammonium acetate of pH 6.0. The absorption spectra of partially purified cytochrome c^{551} and the brown protein are shown in Figs. 1 and 2. In visible regions there were maxima at 551, 521 and 416 mμ in reduced form, and 521 and 411 mμ in oxidized form of cytochrome c^{551}, the ratios of the extinctions of α to β bands and Soret to α bands of the cytochrome are shown in Table I. On the other hand, the brown protein exhibited an absorption spectrum with maximum at 415 mμ in reduced form and 408 mμ in oxidized form, and apparently no other band has detectable in visible region.

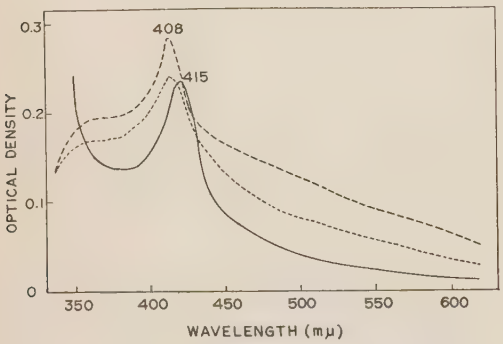

FIG. 2. Absorption spectra of the brown protein. ——: Reduced form ($Na_2S_2O_4$), -----: Oxidized form, ⋯: Oxidized form+KCN.

TABLE I

Spectroscopic and Physicochemical Properties of Cytochrome c^{551} and the Brown Protein

	cytochrome c^{551}	brown protein
Absorption Maxima (mμ)		
oxidized form	521 411	408
reduced form	551 521 416	415
pyridine-heme (red.)	550 520 415	—
Extinction Ratio		
E551(R)/E521 (R)	1.4	
E418 (R)/E551 (R)	6.2	
$S_{20,w}$	1.3S	1.6S
U (anodic mobility)	10.9×10^{-5}cm.^{2} sec^{-1} volt.$^{-1}$	
E_0'	+0.249 volt.	+0.360 volt.
Autoxidizability	none	none

Sedimentation Constants of Cytochrome c^{551} and the Brown Protein—Measurements of sedimentation constant were performed at a single concentration of 0.36 per cent for cytochrome c^{551} and of 0.45 per cent for the brown protein in 0.05 M phosphate buffer, pH 7.0. The sedimentation diagram showed a single sedimentating boundary for each chromoprotein and the sedimentation constants of cytochrome c^{551} and the brown protein were 1.3 S and 1.6 S, respectively.

Electrophoretic Mobilities of Cytochrome c^{551} and the Brown Protein—Cytochrome c^{551} showed a single schlieren peak, while the schlieren pattern of the brown protein indicated the existence of two components which consisted of a faster moving brown colored component and a slower moving colorless impurity. Electrophoretic mobility of 10.9×10^{-5}cm.^{2} sec^{-1}volt.$^{-1}$ was obtained for cytochrome c^{551}.

Oxidation-reduction Potentials of Cytochrome c^{551} and the Brown Protein—Oxidation-reduction potentials for these chromoproteins were measured at 25°C and at pH 7.0. The E_0' of cytochrome c^{551} was +0.249 volt. while that for the brown protein was +0.36 volt. These values were caluclated from the data shown in Figs. 3 and 4 by means of the following equation, where the value 0.43 is the mid-point potential of ferricyanide-ferrocyanide system (*2*), and E_0' is the mid-point potential of cytochrome c^{551} or the brown protein systems.

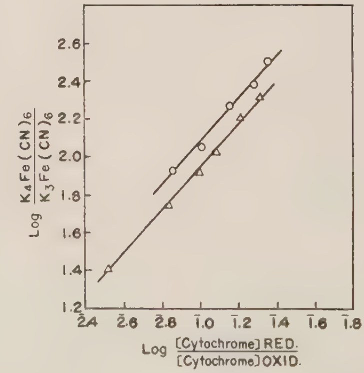

FIG. 3. Determinations of the oxidation-reduction potential of cytochrome c^{551} (○) and mammalian cytochrome c (△).

Study carried out at 25°C at pH 7.0 in the presence of the ferricyanide-ferrocyanide system.

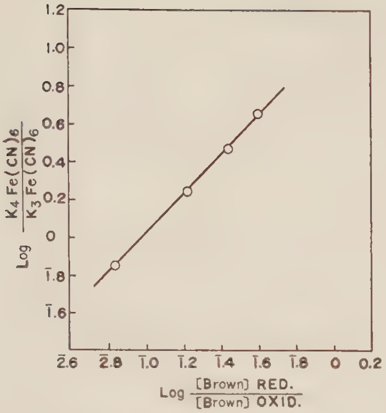

FIG. 4. Determination of the oxidation-reduction potential of the brown protein.

Study carried out at 25°C at pH 7.0 in the presence of the ferricyanide-ferrocyanide system.

$$E_0'(\text{cytochrome}) = 0.43 - 0.059 \log \frac{K_4Fe(CN)_6}{K_3Fe(CN)_6} \times \frac{\text{ferricytochrome}}{\text{ferrocytochrome}}$$

Heme Group of Cytochrome c^{551}—Cytochrome c^{551} exhibited a typical c-type pyridine hemochromogen spectrum, in reduced form there were maxima at 550, 520 and 415 mμ, and futhermore, the prosthetic group could not be split from the protein by the treatment with HCl-acetone.

Chemical Nature of the Brown Protein—As yet no detailed analysis has been made of iron and other metals in brown protein, but no evidence for the presence of heme group has been obtained in the protein so far.

No shift of the absorption band at 408 mμ of oxidized form occurred on addition of potassium cyanide but the absorbancy diminished moderately (Fig. 2). Neither oxidized nor reduced form of the brown protein did combine with carbon monoxide. These results suggested that this chromoprotein was different from the pigment called 'mitochrome' which was found in liver mitochondria, a hemeprotein derived from the preparations of cytochrome $(a+a_3)$ and of cytochrome b (4), although the spectrum of the brown protein was in a close resemblance to mitochrome.

Enzymatic Properties of Cytochrome c^{551} and the Brown Protein—As was mentioned in the previous paper, cytochrome c^{551} and the brown protein were isolated from the *Micrococcus* grown in aerobic and in anaerobic conditions with nitrate. Under anaerobic condition, the absolute amount of cytochrome c^{551} was reduced (*1*), whereas that of the brown protein remained unchanged. The greater part of cytochrome c^{551} was found in subcellular particles from which it was extracted with 40 per cent of acetone without difficulty. Cytochrome c^{551} was easily reduced by succinate, formate and DPNH and was reoxidized by oxygen in the presence of the extracts of this *Micrococcus*. The initial rate of enzymatic reduction of this cytochrome was comparable to those of cytochromes b_4 (I) and (II) while the initial rate of reoxidation of reduced cytochrome c^{551} by oxygen was faster than those of cytochromes b_4 (I) and b_4 (II). It was comfermed the that reduced brown protein was capable of functioning as an electron donor in the cytochrome oxidase system in place of cytochromes.

DISCUSSION

Five chromoproteins have been isolated from a halotolerant denitrifying *Micrococcus* and have been considerably purified by chromatography on DEAE-cellulose column. Cytochrome c^{551} and the brown protein were, however, less acidic than the other cytochromes, therefore a simple repetition of chromatography on DEAE-cellulose column was not so useful as expected for the purification of these chromoproteins. Precipitation with ammonium sulfate and electrophoresis with starch zone were available for further purification of cytochrome c^{551} but the brown protein seemed to break down during these procedures. Cytochrome c^{551} and the brown protein used in their characterizations were of the purity of 80 per cent and 60 per cent, respectively. As was shown in Fig. 1. and Table I, cytochrome c^{551} resembled closely mammalian cytochrome c in spectroscopic property and in the oxidation-reduction potential but seemed to have a lower isoelectric point than mammalian cytochrome c. A number of different cytochormes of type-c similar to cytochrome c^{551} in spectroscopic and physicochemical properties have been isolated from a nitrogen fixing bacteria, *Azotobacter vinelandii* (*5*, *6*) and also from denitrifying bacteria, *Micrococcus denitrificans* (*7*, *8*), *Pseudomonas denitrificans* (*7*, *8*, *12*) and *Pseudomonas aeruginosa* (*9*). These nitrogen fixing and denitrifying bacteria have been shown to contain several different cytochromes in addition to a cytochrome resembled cytochrome c^{551} of the *Micrococcus*, and recently a number of cytochromes of type c and even of type b (*Micrococcus denitrificans and Pseudomonas denitificans*) were isolated from these bacteria (*5*, *6*, *8–11*).

In these bacteria, interrelationship of a number of different cytochromes in oxygen and nitrate respiration systems and in nitrogen fixation system which also has been supposed as a form of respiration (*13*) are not yet clear.

Cytochrome c^{551} was easily reduced by succinate, formate and DPNH and was reoxidized by oxygen in the presence of an extract of this *Micrococcus*. As was described in the paragraph treated upon enzymatic properties of cytochrome c^{551} and the brown protein, the initial rate of enzymatic reduction of cytochrome c^{551} was comparable to those of cytochromes b_4 (I) and b_4 (II) while the initial rate of reoxidation of reduced cytochrome c^{551} by oxygen was fatser than to those for cytochromes b_4 (I) and b_4 (II).

It suggests that cytochrome c^{551} may function a role similar to that of mammalian cytochrome c. Interralationship of cytochrome 560*, cytochromes b_4 (I) and b_4 (II), cytochrome c^{551} and the brown protein participating in nitrate and oxygen respirations is not yet clear at present. As was shown in Fig. 7 of the previous paper (*1*), however, it is probable that cytochrome 560, cytochromes b_4 (I), b_4(II) and c^{551} function as intermediate electron carriers in the oxygen respiration system while in nitrate respiration system cytochrome 560 and b_4 (I) participate in carrying electron from succinate, formate and DPNH toward nitrate, nitrite and other dissimilatory nitrate reduction products get to nitrogen gas. The respiratory chain of this *Micrococcus* had been presented by Chance (*14*), in which cytochromes b_4 (I) and b_4 (II) and CO-binding pigment were included as terminal respiratory pigment.

Physiological functions of HdR and the brown protein remained to be determined. Recently, however HdR was confirmed to function as soluble factor in particulate nitrite reductase system (*15*).

SUMMARY

Cytochrome c^{551} and a brown protein have been obtained from a denitryfying *Micrococcus* together with other cytochromes, that is, cytochromes b_4(I), b_4(II) and cytochrome 625, 553 (HdR).

Cytochrome c^{551} resembled closely mammalian cytochrme c in spectroscopic and in some other properties but seemed to have a lower isoelectric poiet than mammalian cytochrome c.

Chemical nature of the brown protein is not yet clear, and no evidence for the presence of heme group has been obtained in this chromoprotein.

The authors wishes to express his gratitude to Prof. F. Egami and Dr. S. Taniguchi for their valuable and encouraging advices and to Mr. E. Itagaki for his discussion. The author also express his thanks to Nagoya Factory of Fujisawa Chemicals Co. Ltd. for the cultivation of the bacteria.

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* Most recently it was isolated from the *Micrococcus* by use of sodium deoxycholate and lipase (*1*).

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NOTE ADDED IN PROOF

It has been recently confirmed that, among the respiratory pigments obtained in soluble forms, the reduced brown protein was the best electron donor to the nitrate reductase (NaR) also isolated from this *Micrococcus*. The NaR was purified by chromatography on DEAE-cellulose column free of cytochromes and cytochrome reductase and oxidase. The initial rate of reoxidation of the reduced brown protein by nitrate was greater than that by oxygen in the presence of the *Micrococcus* particulate fraction; $+\Delta$ O. D. (at 408mμ). min.$^{-1}$mg. Nitrogen^{-1}, 0.210(NO_3^-) and 0.56 (O_2), respectively. These results strongly indicate that the brown protein may function as a direct electron donor to the NaR.

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Collagenase Digestion of Collagen

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It has been well known that soluble collagen could be reprecipitated as a fibrous material by a change of pH of the solution under a controlled ionic strength, and that the reprecipitated fibers possess the characteristic striations with a period of 650 to 700 Angstrom when examined with an electron microscope (*1*) and x-ray diffraction (*2*). It is obvious that this characteristic nature of collagen fibers would firmly be connected with the primary and higher order structure of the collagen molecule. Therefore, in order to elucidate the mechanism of the reconstitution of collagen fibers, it is necessary to investigate the characteristic structure in collagen molecule which is expected to contribute to the reconstitution of the periodic structure. For this purpose, it was attempted to apply mild and specific methods such as enzymatic digestion for the study of the characteristic structure in collagen molecule.

It has been established that collagenase is a proteinase with a very high degree of substrate specificity and attacks only collagen and gelatin among proteins (*3*, *4*). It could, therefore, be considered that the specificity of collagenase would directly be connected with the characteristic structure of collagen. Thus, the nature of collagenase was deduced from the results obtained by the analysis of the digestion products of collagen with collagenase, then the presumed substrate specificity of collagenase was verified with the use of peptides of known structure and then the primary structure of collagen molecule was constructed in view of the substrate specificity of the enzyme.

In the present report, a purification method of collagenase and analysis of the end product of the hydrolysis of collagen with this enzyme are described. The specificity of collagenase and the structure of collagen are also discussed.

EXPERIMENTAL

Preparation of Collagen—Collagen was prepared from rat tail tendon as described by Noda (*5*) with the following modifications. The collagen solution extracted with 0.05% acetic acid was precipitated with 0.1 *M* citrate buffer of pH between 4.8 and 5.0 and keeping the temperature at 20°C for a few hours. The precipitate thus obtained was dissolved in 0.05% acetic acid, and then precipitation was performed with citrate buffer in the same way as before. This procedure of precipitation and solution was repeated three times.

Preparation of Crude Enzyme—The enzyme used in this study was prepared from the culture medium of *Clostridium histolyticum* strain H_{22} supplied by Dr. Shonosuke Miyazaki of the Institute for Infectious Diseases, University of Tokyo. The organism was cultivated by the method of Monier *et al.* (*6*). After a period of growth 40 to 42 hours at 37°C, the culture was passed through a bacteriological filter. The crude enzyme was prepared by ammonium sulfate fractionation as described by Mandl *et al.* (*7*). Thus, a fraction precipitated between 22 and 30% ammonium sulfate concentration was lyophilized after dialyzing against water.

Assay Method—In all cases the activity of collagenase was routinely determined as follows. To 0.1% soluble collagen of rat tail tendon in 0.05% acetic acid, equal volume of 0.1 *M* borate buffer containing 2×10^{-3} *M* calcium chloride was added and the pH of the solution was adjusted to pH 7.5 with *N* NaOH. At that time, the collagen became cord-like fiber and floated in the solution. To 2 ml. of collagen suspension in the buffer, a suitable predetermined amount of collagenase was added. The reaction mixture was kept at 20°C and the time required to clarify the collagen suspension was measured This method is a simple and easy way of determining the relative activities of the enzyme fractions in the course of purification. However, in the case of zone electrophoresis, the following methods were applied to each section to look for the presence of collagenase. 20 mg. of azocoll (*8*) was suspended in 0.05 *M* borate buffer containing 2×10^{-3} *M* calcium chloride at the pH of 7.5 and an aliquot of the filtrate from each section was added. The reaction mixture was kept at 37°C for one hour with occassional shaking. Then the remaining azocoll which was not solubilized was filtered off immediately and colorimetry was made with a Kotaki model AKA 5-D colorimeter at the wave length of 520 mμ. To 2 ml. of 0.6% casein in the same buffer mentioned above, an aliquot of the filtrate from each section was added. The reaction mixture was kept at 37°C for 20 minutes. After the end of incubation, 2 ml. of 0.44 *M* trichloroacetic acid solution was added and the solution was left over 10 minutes at the same temperature. After filtrating the insoluble material with filter paper, colorimetry was made with a Beckman model DU spectrophotometer at the wave length of 275 mμ. By using these two methods, the section which attacked the azocoll, but was not active against the casein was selected. In the selected sections, the collagenase activity was ascertained with collagen suspension as the substrate as described above.

Preparation of Collagen Digest—To 62 mg. of heat-denatured (70°C, 10 minutes) collagen in 25.5 ml. of 0.05% acetic acid, 23.5 ml. of 0.1 *M* borate buffer of pH 7.4 containing 2×10^{-3} *M* calcium chloride was added and the pH of the solution was adjusted to pH 7.4 with 2 *N* NaOH (3 drops). This collagen solution was incubated at 37°C for 6 hours with 0.0032 per cent of enzyme which was enough for complete digestion of collagen in 6 hours. At the end of incubation, the reaction was interrupted by adjusting the pH to 2.2 with 6 *N* hydrochloric acid. Aliquots of the reaction mixture, 0.1 ml. each, were withdrawn to use for the assay of total amino increments, and the remaining solution was immediately applied to the column.

Fractionation of Peptides—The chromatography of collagen digest was performed with a column (150× 0.9 cm.) of Dowex 50–X2 according to the method of Hirs *et al.* (*9*). The method of preparing and operating the column was the same as the procedure by Moore *et al.* (*10*). The mixing chamber for obtaining a gradual change in pH and ionic strength had a volume of 600 ml. and the initial flow rate was 7 ml. per hour. The mixing device was attached after 0.71 effluent liter and the mixing chamber was filled with *N* sodium acetate-citrate buffer at the pH of 5.1. A 2 *N* buffer was substituted after 1.31 effluent liters. After 1.6 effluent liters the temperature was raised to 50°C and a 2 *N* sodium acetate buffer at the pH of 6.6 was introduced directly.

The effluents from the column were collected in 2 ml. fractions and 0.5 ml. aliquot of each fraction was analyzed by trinitrophenylation method (*11*). The results obtained were shown in Fig. 3.

Chemical Methods—Total amino increments were determined by trinitrophenylation method (TNP-method) according to the procedure of Satake *et al.* (*11*). After 0.1 ml. of the digest was diluted with water to 0.5 ml., 2 ml. of 0.5 *M* disodium phosphate was added to the solution and the pH was adjusted to pH 8.0 with 3 *N* hydrochloric acid. The alkaline reaction mixture thus prepared was incubated at 40°C for 90 minutes with 2 ml. of 0.1% 2, 4, 6-trinitrobenzene 1-sulfonic acid (TNBS) in a dark room and then acidified with 1 ml. of 6 *N* hydrochloric acid. Colorimetry was made with a Beckman model DU spectrophotometer at the wave length of 340 mμ. Analysis of effluent fractions obtained by column chromatography was also performed with TNP-method as described above. For this purpose 0.5 ml. of each fraction was used.

Amino acid composition of each fraction was determined by dinitrophenylation method (DNP-method) (*12*) and the C-terminal amino acid residues were determined by hydrazinolysis method according to the procedure of Akabori *et al.* (*13*).

RESULTS

Purification of collagenase—For the purpose of purification of collagenase, starch zone electrophoresis as described by Kunkel (*14*) was used. Washed starch was suspended in a 0.05 *M* Veronal buffer containing 5×10^{-3} *M* calcium chloride at the pH of 8.6 and a slab having dimensions of 40×2.5×1 cm. was prepared. 100 to 200 mg. of crude enzyme in the same buffer solution was mixed with dried starch and applied at the center of the slab. Electrophoresis was carried out for 36 to 40 hours at 4°C with a constant voltage of 250 volts and a current of about 10 mA. At the end of the time, protein fractions migrated were located by developing a strip of filter paper printed on the surface of the slab with bromphenolblue. Then the slab was divided into 1 cm. segments on either side of the center. Each segment was shaken with 5 ml. of 0.05 *M* borate buffer containing

10^{-3} *M* calcium chloride at the pH of 7.5 and the starch was filtered off. A 0.2 ml. aliquot of each fraction was used for the assay of azocoll digestion and casein digestion respectively. The results obtained from these assays were shown in Fig. 1.

It was found that the collagenase fraction which was active for azocoll but inactive for casein migrated toward the anode and was located at the distance of 12 to 15 cm. from the center. The pigment was found to migrate in the opposite direction. The fractions migrating between 12 and 15 cm. toward the anode were collected and lyophilized after dialyzing against distilled water. Thus 20 to 30 mg. of purified collagenase was obtained from 10 liters of *Cl. histolyticum* culture filtrates.

Properties of the Purified Collagenase—This enzyme preparation was inactive against the following synthetic peptides and proteins: leucineamide, benzoylarginineamide, glycylglycine, tryglycine, alanylglycylglycine, glycylprolylleucine, prolylleucylglycine, polyleucine, casein and denatured serum albumin. It is only active against native collagen and its derivatives such as gelatin and azocoll among the substrates tested. For these studies the synthetic peptides were each dissolved in 0.05 *M* borate buffer of pH 7.5 and incubated with the enzyme solution in an amount of 50 μg. per ml. of reaction mixture for 4 hours at 37°C. Paper chromatography and paper electrophoresis followed by ninhydrin reaction were employed for the assay of hydrolytic activity. In the case of protein substrate, hydrolytic activity was assayed by the method of spectrophotometry at 275 mμ. of trichloroacetic acid soluble fraction.

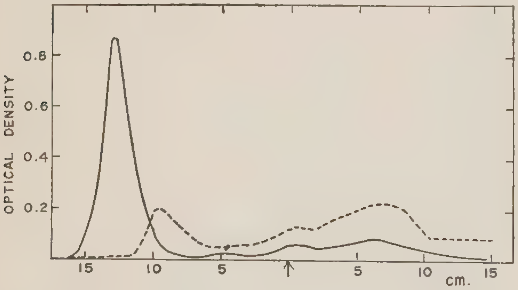

FIG. 1. The relation of proteinase activity to the distance of migration in starch electrophoretic separation.

The arrow indicates the origin. The activity for azocoll (solid line) and that for casein (dotted line) were indicated.

Analysis of End Groups of the Digest—The results obtained from the analyses of N- and C- terminal increments of the collagen digests in the course of hydrolysis by the action of collagenase at pH 7.5 and 37°C were shown in Table I and II.

The determination of the N-terminal residues of the digest was carried out by dinitrophenylation followed by hydrolysis in a sealed tube with 6 *N* hydrochloric acid at 105°C for 8 hours and then by paper chromatography. For the determination of C-terminal residues, aliquots of the reaction mixture were immediately lyophilized at regular intervals of time and the resultant material containing 25 mg. of the collagen digest was subjected to hydrazinolysis followed by dinitrophenylation and paper chromatography. The identity of the amino acid component of the DNP-derivative was further established by identifying the free amino acids liberated from the hydrolysis of the DNP-derivative in a sealed tube with 29% aqueous ammonia for 6 to 10 hours at 100°C. In each case a control experiment was performed by the same way using buffer solution in place of the enzyme. The values of both terminal residues in Table I and II were not corrected for hydrolytic and other losses.

TABLE I

N-terminal Residues of Products Resulting from Digestion of Collagen by Collagenase

N-terminal residue	Control μ mole/10 mg.	30 min. reaction μmole/10 mg.
Glycine	trace	0.97
Aspartic acid	0.01	0.04
Serine	trace	trace
Valine	trace	trace

From the results shown in Table I, it can be seen that only glycine residue increased among the N-terminal residues during

TABLE II

C-terminal Residues of Products Resulting from Digestion of Collagen by Collagenase

C-terminal residue	Control µmole/25 mg.	30 min. reaction µmole/25 mg.	4 hours reaction µmole/25 mg.
Glycine	0.05	0.03	0.04
Serine	0.02	0.03	0.02
Aspartic and/or Glutamic acid	0.08	0.09	—
Alanine	0.01	0.26	0.52
Hydroxyproline	0.00	1.01	1.81

the course of the hydrolysis, and it accounted for more than 95 per cent of total moles of the N-termini recovered after 30 minutes incubation. It was also found that a small amount of aspartic acid appeared during the incubation. No free amino acid was detected in the collagen digest with collagenase. As regards the C-terminal residues, it was found as shown in Table II that only two amino acids, alanine and hydroxyproline, increased and other amino acids remained in approximately the same amounts within the limits of an experimental error even after 4 hours incubation. It can also be seen that the amounts of alanine and hydroxyproline in the C-termini acounted for each 16 to 18 and 55 to 70 per cent of the total moles of the C-termini of the digest recovered after incubation with collagenase.

Fraction of Collagen Digest—The course of the collagenolytic hydrolysis of heat-denatured collagen at pH 7.5 and 37°C is shown in Fig. 2. The curve is corrected for the almost negligible amount of ninhydrin color contributed by the autolysis of the collagenase during digestion. It was found that the hydrolysis proceeded almost to completion within a period of approximately 2 hours and that the end products after 6 hours incubation had an average molecular weight of about 600 to 700. The same value of molecular weight of end products was obtained by TNP-method (*11*). From the results shown in Fig. 2, it was concluded that the digestion of heat-denatured collagen by collagen by collagenase was complete after 6 hours incubation.

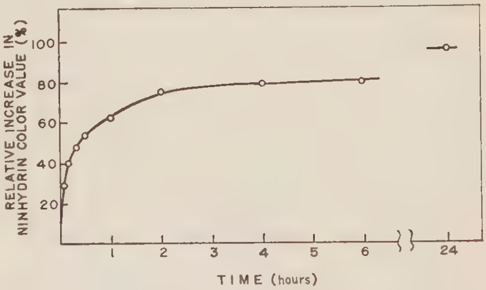

FIG. 2. The rate of the hydrolysis of soluble collagen by purified collagenase at pH 7.5 and 37°C.

0.08 per cent of heat-denatured collagen was hydrolyzed with 0.0032 per cent of collagenase.

The separation of the collagen digest was performed with a column of Dowex 50-X2 according to the method of Hirs *et al.* (*9*). When the digestion products obtained after 6 hours of collagenolytic hydrolysis of 62 mg. of heat-denatured collagen were chromatographed on 150×0.9 cm. column of Dowex 50-X2, the effluent curve obtained was shown in Fig. 3. The chromatogram in Fig. 3 showed that the digest could be fractionated into approximately 30 peaks iucluding the small peaks which was not numbered. At 1.9 liters, the resin was removed from the column and washed with 0.2 *N* NaOH and it was found that about 5 per cent of the total moles of the digest used was firmly bound to the resin as peptides.

The molar recovery of the total peptides eluted as calculated from the determination of end groups by TNP-method (*11*) was 94 per cent of the digest used. The molar recovery of each peak obtained by the same

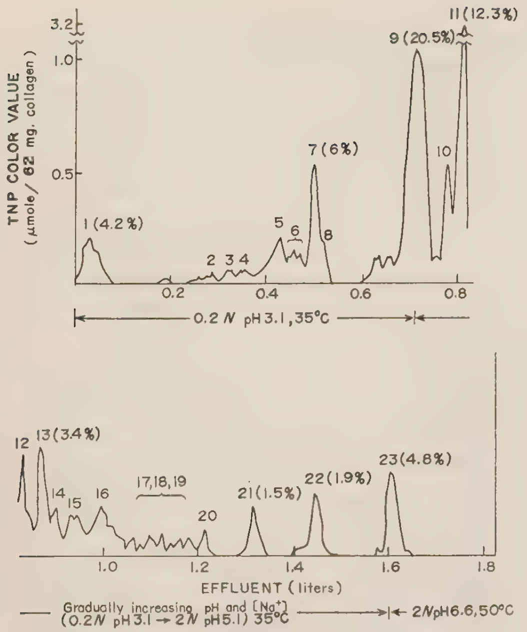

FIG. 3. Chromatography of peptides in a 6 hours collagenolytic digest of soluble collagen. A digest from 62mg. of protein was chromatographed on a 150×0.9 cm. column of Dowex 50-X2. The mixing chamber for the in fluent buffer had a volume of 600 ml. The ef fluent was collected in 2 ml. fractions and 0.5 ml. aliquots were withdrawn for analysis by TNP-method.

way is given in a parenthesis adjacent to the number of corresponding peak.

End groups of peaks 9 to 13 were qualitatively investigated by means of DNP-method for N-terminal residues and hydrazinolysis for C-terminals. It was found that the N-terminal residues of four peaks expect peak 10 were all glycine and that of peak 10 was a mixture of glycine and aspartic acid. As regards the C-terminal residues, hydroxyproline was found to be predominant in these four peaks except peak 11 and small amounts of serine, glycine, threonine, alanine and aspartic and/or glutamic acid were found to be contaminating in the peaks. However, the main part of the C-termini of peak 11 was identified as alanine residue and minor part hydroxyproline.

Estimation of Two Peptides and Their Structural Studies—Two main peaks 9 and 11 in Fig. 3 were further fractionated by paper chromatography after dinitrophenylation (*12*). DNP-derivatives of peptides in these peaks were all extracted into acidic ethyl acetate. By two-dimensional paper chromatography using both toluene-chloroethanol-pyridine-aqueous ammonia and 1.5 M phosphate buffer of pH 6.0, it was found that peaks 9 and 11 were composed of three peptides respectively. The amount of main peptide (F-9A) of peak 9 as calculated from the optical density of DNP-derivative accounted for 78 per cent of the total moles of peak 9 and that of main peptide (F-11A) of peak 11 as calculated by the same way accounted for 73 per cent of the total moles of peak 11.

The N-terminal residues and the sizes of the main peptides in peak 9 and 11 were determined by the hydrolysis of DNP-peptides in 6 N hydrochloric acid in sealed tubes at 105°C for 8 hours respectively. The N-terminal residues were identified and estimated quantitatively as DNP-amino acids recovered from hydrolysates by means of two-dimensional ascending paper chromatography using the same solvent systems as described above. Free amino acids from the hydrolysates were quantitatively analyzed by DNP-method described above. The C-terminal residues were identified by hydrazinolysis (*13*).

The results of the analysis of each peptide and its constituent amino acids are given in Table III. It is evident from Table III that each peptide was composed of amino acids in equimolar proportions and that F-9A (main peptide in peak 9) was glycylprolylhydroxyproline and F-11A (main peptide in peak 11) was glycylprolylalanine. The amounts of F-9A and F-11A accounted for 16 per cent and 9 per cent respectively of total moles of collagen digest recovered. It can, therefore, be concluded that the tripeptides in type of G-P-R (G: glycine, P: proline and R: almost any amino acid) acounted for 25 per cent of total moles recovered.

0.25 micromoles of DNP-derivative of each fraction was used for the analysis. The results were expressed in micromoles and in

Ttable III

Results of Amino Acid Analysis of Eeach Main Component of Peak 9 and 11

0.25 micromoles of DNP-derivative of each fraction were used for the analysis. The results were expressed in micromoles and in per cent of the sampled micromole recovered.

Fraction	F-9A μmole recovered (%)			F-11A μmole recovered (%)		
N-terminal	Glycine	0.19	76	Glycine	0.18	72
Middle	Proline	0.25	100	Proline	0.23	92
C-terminal	Hydroxyproline	0.21	84	Alanine	0.19	76

per cent of the sampled micromole recovered.

DISCUSSION

Recently several reports concerning the analysis of the hydrolysates of collagen by partial acidic and alkaline hydrolysis and by collagenase have appeared (*4*, *15–19*). One may be able to draw a general conclusion from these results that collagenase attacks the bond of -X. Gly- in collagen molecule with the result of the release of glycine residue in the N-termini and that of X-residue in the C-termini. X stands for almost any kind of amino acid residues. It can be further seen that the amino acid sequence in the type of G-P-R is frequently present in collagen molecule (*18*, *19*) and tripeptides of that type are released in collagen digests after the action of collagenase (*17*).

Ogle *et al.* isolated two tripeptides, glycylprolylhydroxyproline and glycylprolylalanine, in a large quantities from the collagenase digest of cattle Achilles tendon collagen (*17*). In the present study it was found that the same two tripeptides were released from rat tail tendon collagen by the action of purified collagenase and that the percentage of each amino acid in collagen which is included in the two tripeptides isolated are; glycine 11, proline 28, hydroxyproline 24 and and alanine 13 respectively based on the amino acid content of the collagen used (20). These values are in agreement with all the findings mentioned above.

When collagen was incubated with collagenase, the number average molecular weight of the digest decreased to about 600 to 700. Seifter *et al.*, while studying the action of collagenase on ichthyocol and its gelatin, obtained the end products with approximately 500 in molecular weight (*4*). These results indicate that a large amount of tripeptides in the type of G-P-R are released in the course of collagen digestion with collagenase.

However, in a preliminary study of the analysis of each peak in Fig. 3, it was found that peaks 13, 14, 22, and 23 were all composed of more than 10 amino acid residues including proline residue less than one-third of the total number of residues in the peptides respectively and that total amount of these long peptides were more than 10 per cent of the total number of moles of the collagen digest recovered. This fact suggests that collagen molecule is partly composed of rather long peptides unsusceptible to the action of cllagenase in addition to the peptides of the sequence susceptible to the action of the enzyme. This is consistent with the assumption used by Bear (*2*) and with the results obtained by Mandl *et al.* (*16*). The tripeptide structure of the so-called crystalline portion of the collagen molecule agrees with the model of Ramachandran (*21*) and Rich and Crick (*22*) from their x-ray data.

It is worth further to note that these characteristic amino acid sequence in the collagen molecule closely related to the substrate specificity of collagenase is undoubtedly responsible for the striations of the collagen fiber (*23*).

The substrate specificity of collagenase might closely be connected with the particular sequence of amino acids in collagen molecule such as -Gly Pro- or more limited sequence in the type of G-P-R. Fromthese considerations and also from the accumulated informations (*3*, *4*, *6*) of oligopeptides unsusceptible to the action of collagenase, the study of the substrate specificity of the enzyme is now carried on with synthetic peptides which may possibly be a substrate.

Further studies on the action of collagenase on synthetic peptides and the detail of the analysis of collagen digest by the enzyme will be reported in subsequent communications.

SUMMARY

1. *Clostridium hystolyticum* collagenase has been purified by use of ammonium sulfate fractionation and starch zone electrophoresis. Purified collagenase has been found to act only on collagen and gelatin and to be free from non specific proteinases and peptidases.

2. From the N- and C-terminal amino acid analysis of collagenase digest of collagen, glycine was found in more than 95 per cent of the N-termini and hydroxyproline and alanine in 55 to 70 per cent and 16 to 18 per cent respectively of the C-termini.

3. Enzymatic digest of collagen was resolved in to about 30 fractions by Dowex 50-X2 column chromatography. Two main fractions were identified as glycylprolylhydroxyproline and glycylprolylalanine respectively, and both these peptides accounted for about 25 per cent of the total mole recovery in the digest.

The auther wishes to express his thanks to Prof. H. Noda of the Department for his helpful discussion and encouragement throughout this work. The author is indebted to Dr. J. Koyama of the Department for his kind guidance and discussion in the work of collagenase preparation and to Dr. S. Miyazaki of the Institute for Infectious Diseases, University of Tokyo, for the supply of *Clostridium histolyticum* strain H_{22}.

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Specificity of Collagenase*

The Action of Collagenase on Synthetic Peptides

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In the previous paper (*1*) a purification method of collagenase from *Clostridium histolyticum* and the analysis of the products resulting from the action of collagenase on the acid soluble collagen of rat tail tendon were reported, and the chemical structure of collagen in connection with the specificity of collagenase was discussed.

With regards to the specificity of collagenase, effort has been made to look for a possible synthetic substrate for collagenase on the basis of the accumulated informations (*1–6*) from the analysis of the products from collagen digested by collagenase. Several short reports have appeared recently (*7–11*).

In the present communication the effect of collagenase on a synthetic polymer *i. e.* poly-(L-prolyl-L-leucyl-glycyl) (*12*) is described. The action of collagenase on the pentapeptide amide, which was synthesized as a possible simplest substrate is also described, and some discussion of the nature of the action of collagenase is made.

EXPERIMENTALS

Preparation of Enzyme—The purification method of the enzyme has been described previously (*1*).

Synthetic Peptide Used as the Substrate—Most of the synthetic compounds used here were synthesized as described previously (*13*). The sample of poly-(L-prolyl-L-leucyl-glycyl) was furnished by Dr. Shumpei Sakakibara of the Institute for Protein Research, Osaka University.

Collagenase Digestion of the Polymer—Fifty mg. of poly-(L-prolyl-L-leucyl-glycyl) of molecular weight of 3,000, was dissolved in a small amount of dimethylformamide while heating and then diluted with 10 volumes or more of distilled water. After the precipitate which appeared was filtered off, the supernatant was dried up *in vacuo*. About 0.1 μmole of the water soluble polymer thus obtained was dissolved in 5 ml. of 0.05 M borate buffer containing 10^{-3} M $CaCl_2$ at pH 7.4 and incubated at 37°C for 4 hours with 100 μg. of purified collagenase. Even in cases when the substrate was not dissolved completely, the reaction mixture became clear in the course of digestion. Control experiments were performed with boiled enzyme.

Isolation and Separation of Peptides—The polymer digest was immediately dinitrophenylated by the method of Levy *et al.* (*14*). After the removal with ether of excess DNFB from alkaline reaction mixture, the solution was acidified with concentrated HCl and extracted three times with 5 ml. of ethyl acetate. DNP-derivatives in the reaction mixture were almost completely extracted with the ethyl acetate, and those in control solution were precipitated and floated between the organic and aqueous layer. The ethyl acetate extract was dried up *in vacuo* after a washing with N HCl. Excess dinitrophenol was removed by sublimation. The resultant products were analysed with one-dimensional ascending chromatography on Toyoroshi No. 51 paper using *tert*-amyl alcohol saturated with 0.1 M phthalate buffer pH 5.9 as described by Levy *et al.* (*14*).

Paper Electrophoresis—Paper electrophoresis was carried out using a B type apparatus of Natsume Seisakusho Ltd. Toyoroshi No. 51 paper and 0.25 N acetic acid pH 2.8 were used throughout.

Estimation of Peptides—Each spot of DNP-derivatives on paper chromatogram was cut out and eluted with 4 ml. of 1% sodium bicarbonate. The colorimetry was made with a Beckman model DU spectrophotometer.

Determination of Amino Acid Sequence—Known amounts of DNP-peptides were hydrolyzed in 6 N HCl in sealed tubes at 105°C for 8 to 15 hours and repeatedly evaporated to dryness to remove most of the HCl. The N-terminal residues were identified as DNP-amino acids which were recovered from hydrolysates by means of one-dimensional ascending paper chromatography using the same solvent system as described above. Free amino acids from the hydrolysate were quantitatively analysed by the DNP-method described above. Since the general pattern of amino acid sequence in the polymer had already been known, the actual sequence in a peptide could be established by the determination of the quantities of its constituent amino acids and of terminal amino acids, provided the length of the peptide was no longer than seven or eight residues.

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* Reported in part as a preliminary note in *Biochim. et Biophys. Acta*, **34**, 298 (1959) Abbreviations: DNP, dinitrophenyl; DNFB, dinitrofluorobenzene; DNP-OH, dinitrophenol; G, glycyl; P, prolyl; L, leucyl.

RESULTS

Separation of Peptides—It was found that the suspension of the polymer, poly-(L-prolyl-L-leucyl-glycyl), in borate buffer incubated with collagenase at 37°C became clear after 3 hours, and the main product in the reaction mixture was identified as glycylprolylleucine by means of a chromatographic comparison with an authentic sample.

In Fig. 1, a typical example of the ascending chromatogram of DNP-derivatives obtained from the dinitrophenylated digest is shown. Only three prominent spots were observed. These will be referred to as fractions S-1, S-2 and S-3 respectively. No free amino acid was detected from the digest by paper electrophoresis and by paper chromatography. In the control experiment using boiled enzyme, no appreciable DNP-derivative could be extracted with ethyl acetate from the dinitrophenylated digest. Sometimes the pattern of fraction S-1 overlapped with that of fraction S-2 because of the large quantity of the latter. Rechromatography of the fraction S-1 with ascending procedure was successfully applied to make it free from fraction S-2. The pattern of fraction S-3 was observed at the position overlapped with dinitrophenol. The contaminating dinitrophenol was removed from this fraction by passing through a talc-column.

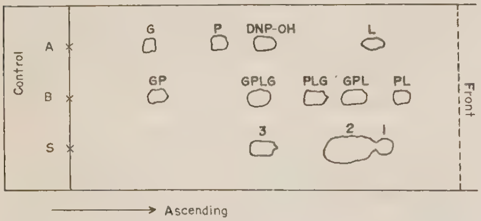

FIG. 1. One-dimensional chromatogram of the DNP-derivatives of the products obtained by the digestion of poly-(L-prolyl-L-leucyl-glycyl) with collagenase.

The solvent system is *tert*-amyl alcohol saturated with 0.1 *M* phthalate buffer of pH 5.9. DNP-derivatives of amino acids and synthetic peptides as control (column A and B) were run in parallel with that of the digest.

Analysis of Peptides and the Estimation of Amino Acid Sequence—The results of the analysis of each peptides and its constituent amino acids are given in Table I and Table II respectively. By the DNP-method, the N-terminal amino acids in fraction S-2 and S-3 were found to be glycine. In the paper chromatograms of the DNP-derivatives

TABLE I

Amounts of Peptides Obtained from the Products of Poly-(L-prolyl-L-leucyl-glycyl) by Collagenase Digestion

Each sample of the peptide was identified as DNP-derivative by paper chromatography (see Fig. 1) and determined after extraction with 1% sodium bicarbonate.

Fraction	Maximum wave length	R-value[3]	μmole recovered	Molar ratio
S-1[1]	375–380	1.07	0.06_2	0.8
S-2	355	0.57	1.56	19
S-3[2]	355	0.58	0.08_2	1.0

1) The spot for S-1 was cut from the paper shown in Fig. 1, and rechromatographed in order to remove a slight contamination from S-2.

2) The DNP-OH was removed from this fraction by passing through a talc column.

3) The ratio of optical density at 390 mμ to that of 360 mμ of DNP-derivatives measured in 1% sodium bicarbonate. This value is characteristic of the DNP-N-terminal group (*14*).

TABLE II

Results of Amino Acid Analysis of Peptides

This table shows the results obtained by the application of DNP-method to free amino acids which remained after the DNP-derivatives were removed from the hydrolysate of each DNP-peptide.

Fraction	μmole used	Glycine		Proline		Leucine	
		μmole	molar ratio	μmole	molar ratio	μmole	molar ratio
S-1	0.05_3	0.04_2	0.8	0.06_3	1.2	0.08_5	1.7
S-2	0.28	trace	0.0	0.25	0.9	0.23	0.8
S-3	0.06_2	0.05_3	0.9	0.05_1	0.8	0.04_3	0.7

recovered from the hydrolysate of each DNP-peptide, small amount of material other than DNP-glycine and which appered by the degradation was detected. Paper chromatograms of the DNP-derivatives from the fraction S-1 showed no spots corresponding to any of possible amino acids, but only a small amount of material resulting possibly from the destruction of a DNP-N-terminal amino acid was present. Therefore, the N-terminal amino acid in fraction S-1 was presumed to be proline. Moreover, the ratio of optical densities at 390 mμ to that at 360 mμ (R-value) (*15*) was 1.07, which was characteristic of the DNP-N-terminal proline. Thus it was concluded that the N-terminus was a proline residue. C-terminal residues of peptides were not determined.

The size of each peptide was estimated from the results obtained by the application of DNP-method to the free amino acid mixture which remained after the DNP-derivatives were removed from the hydrolysate of each DNP-peptide, thus finding the total number of moles of all the amino acid residues. The results obtained are given in Table II.

Since the polymer used in this study was synthesized by the polycondensation of L-prolyl-L-leucyl-glycine by tetraethylpyrophosphate method (*12*), the polymer is expected to have proline in the N-terminal position. From the results shown in Table II, and from the possible sequential structure of the polymer substrate, it is concluded that fraction S-1 was prolylleucylglycylprolylleucine, fraction S-2 was glycylprolylleucine and fraction S-3 was glycylprolylleucylglycine. The molar ratio for these peptide derivatives was 0.8 : 19 : 1.

Since in the original polymer, proline only was in the N-terminus, and after digestion with the enzyme no peptide other than fraction S-1 had a proline residue in the N-terminus, it is evident that fraction S-1 was derived from the N-terminal portion of the original polymer used. Application of a similar line of reasoning on the C-terminal glycine lead us to assume that fraction S-3 was derived from the C-terminal portion of the original polymer.

If the polymer substrate with molecular weight of 3,000 was completely digested by the enzyme, the calculated molar ratio for prolylleucylglycylprolylleucine, glycylprolylleucine and glycylprolylleucylglycine must be 1 : 8.2 : 1. The discrepancy between the observed molar ratio mentioned above and the calculation, may be due to poor recovery of peptides from the ends or due to an error in the determination of the average molecular weight or both.

Digestion of Pentapeptide Amide—Glycyl-L-prolyl-L-leucyl-glycyl-L-proline amide synthesized as a possible simplest substrate for collagenase was digested with the enzyme. For this experiment a reaction mixture was prepared as follows; 1 to 3 mg. of substrate and 100 μg. of collagenase in a total of 5 ml. of 0.05 *M* borate buffer at pH 7.5 and 10^{-3} *M*

$CaCl_2$. From the reaction mixture aliquots were withdrawn after 2 or 4 hours of incubation at 37°C for the analysis by paper electrophoresis and DNP-method. A control mixture containing substrate but no enzyme was incubated and analyzed similarly. In order to identify each spot on the chromatogram, several authentic samples which were expected to be released from the substrate during the enzymatic digestion were run in parallel with the digest. The results of these experiments are shown in Fig. 2 and Fig. 3. By the electrophoretic mobility and the color tone of each spot, spot 1 in Fig. 2 was found to be a mixture of undigested substrate and one of the digested products, glycylprolylleucine, and spot 2 to be a peptide amide, glycylproline amide, which was supposed to be released from the C-terminal portion of the substrate. It was further found that the amount of spot 2 increased in the course of digestion,

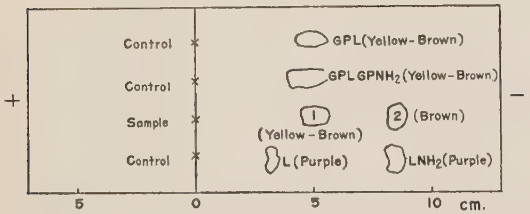

Fig. 2. Paper electrophoretic pattern of the products obtained by the digestron of glycyl-L-prolyl-L-leucyl-glycyl-L-proline amide with collagenase.

Electrophoresis was performed for 45 minutes in 0.25 *N* acetic acid at a current density of 0.2 mA/cm.. Spots of some amino acid, synthetic peptides and amide derivatives are shown in the figure as control and the spots were located with ninhydrin.

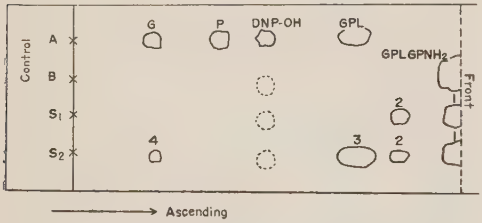

Fig. 3. One-dimensional chromatogram of the DNP-derivatives of the products obtained by the digestion of glycyl-L-prolyl-L-leucyl-glycyl-L-proline amide with collagenase.

The solvent system is identical with that for Fig. 1. Columns A and B are control. Column S_1; DNP-derivatives extracted with alkaline ethyl acetate from dinitrophenylated digest. Column S_2; DNP-derivatives extracted with acidic ethyl acetate from dinitrophenylated digest after washing with alkaline ether.

Both DNP-derivatives of penta- and dipeptide amides were readily extracted with ethyl acetate from the alkaline solution of dinitrophenylated digest as shown in Fig. 3 and some of the DNP-derivative of the dipeptide amide was extracted even with alkaline ether. This undesirable behavior of DNP-derivatives of peptide amides always prevented to recover them in a good yield.

Spot 3 and spot 4 in Fig. 3 were identified as a DNP-derivative of glycylprolylleucine and that of glycylproline respectively, by amino acid analysis after hydrolysis of the extract from the spot. It was found that spot 4 was derived from DNP-glycylproline amide by the cleavage of amide bond in the process of its rechromatogrophy. If glycylproline was released from the N-terminal portion of pentapeptide amide by the action of collagenase, leucylglycylproline amide must be detected from the digest. This tripeptide amide which should have shown purple color by ninhydrin could not be found in the results of paper electrophoresis and direct paper chromatography of the digest. No DNP-leucine was detected among the hydrolysate of each spot in Fig. 3 by paper chromatography. Since no peptides with leucine in the N-terminal position were found in the digest, it is evident that glycylproline of spot 4 in Fig. 3 should be a decomposition product from glycylproline amide which was released from the C-terminal portion of the substrate during the process of dinitrophenylation. It is possible that glycylproline amide was lost from the digest and escaped detection due to the cyclization of the dipeptide amide during the digestion or the analysis of the digest (*9*, *11*, *16*).

From the results mentioned above, it may directly be deduced that pentapeptide amide was very susceptible to the action of collagenase, forming two peptides; glycylprolylleucine and glycylproline amide.

DISCUSSION

From the analysis by x-ray diffraction (*17–20*) and by acidic or basic partial hydrolysis of collagen or gelatin (*21–23*), it has generally been considered that the main portion of the structure of collagen is composed of a single repeating sequence of the type G-P-R (R stands for almost any amino acid residue). The polymer used in this study was synthesized as a model for collagen on the basis of these considerations (*12*). Therefore, it is very interesting to investigate whether the polymer would be susceptible to the action of collagenase or not by the following reasons: whether the polymer of the type G-P-R would be really suitable for a model of collagen or not, and whether the specificity of collagenase would directly and simply be governed by the repeating sequence of the peptide linkage or not. The present work was undertaken on these lines.

Recently Gallop *et al.* suggested (*5*) that "a large sequence of amino acid residues would contribute to the specificity of collagenase" and that "the presence of proline or hydroxyproline in a position penultimate to a glycine residue is a partial requisite of the specificity." From the results obtained in the present study, it was found that the specificity of the enzyme appears to be connected with the repeating sequence of the type G-P-R and that the peptide bond between G and R of the sequence is cleaved by the enzyme. These facts suggest the following possibilities; firstly, the enzyme requires the presence of -Gly. Pro- for its action and splits the bond between X and glycine in the sequence of -X. Gly. Pro-, secondly, the enzyme requires the presence of the same sequence of -Gly. Pro- and splits the bond between glycine and Y in the sequence -Gly. Pro. Y. Gly-, and lastly, the presence of two proline residues in the sequence -Pro. R. Gly. Pro- is required for the action of the enzyme. X, Y and R may be almost any kind of amino acid residues.

Among the products of polymer digestion with collagenase, only a pentapeptide, *i. e.* prolylleucylglycylprolylleucine was derived from the N-terminal portion of the polymer. If the first possibility for the substrate requirement is to be followed, prolylleucine which should be released from the N-terminal portion must be detected in the digested products. No evidence of the appearance of prolylleucine was found by direct paper chromatography of the digest and by DNP-method.

If the second possibility is to be followed, free glycine from the C-terminal portion must be detected in the digested products. Only a tetrapeptide, *i. e.* glycylprolylleucylglycine could be found in the digest, which was released from the C-terminal portion. No glycine was found.

Since no glycine and prolylleucine were found among the digested products, and the pentapeptide from the N-terminal portion and the tetrapeptide from the C-terminal portion were found in comparable amounts, it appeared that collagenase did not attack prolylleucylglycylprolylleucine, in which the imino group of the N-terminal proline is unmasked. It can further be concluded that the presence of an imino acid only in a position penultimate to glycine does not satisfy the specificity requirement of collagenase, and that two proline residues in the sequence -Pro. R. R'. Pro- are required.

Glycyl-L-prolyl-L-leucyl-glycyl-L-proline amide, which should be the simplest substrate for collagenase from the above consideration, was almost completely split after 4-hour incubation with collagenase into glycylprolylleucine and glycylproline amide. These results are consistent with the conclusion that the sequence -Pro. R. R'. Pro- is necessary in a substrate for collagenase.

It is well known, from the analysis of collagen digest with collagenase (*1*, *2*, *5–7*), that collagenase split the peptide bonds involving glycine with little regard to the nature of the preceding amino acid residue, thus resulting many kinds of amino acid in the

C-terminal position of the digest in contrast to the fact that the N-terminal residues are predominantly glycine. It has also been reported that large amounts of two tripeptides *i. e.* glycylprolylhydroxyproline and glycylprolylalanine were isolated from the digest (*1*, *4*). These results go in parallel with the results of the present study in view of the substrate specificity of collagenase. Thus, for R' residue in the scheme -Pro. R. R' Pro-, glycine appears to be most desirable. However, there are evidences that alanine may also be allowed* (*6*, *7*).

Although no leucine could be found for the C-terminal position in the collagen digest (*1*, *5*, *7*), the peptide bond between leucine and glycine in the polymer was cleaved by collagenase. It appears, therefore, that almost any amino acid may be allowed for R residue in the scheme.

Other possibilities than those three listed in the above are, however, not yet completely excluded and several results which could not be explained with the scheme mentioned above were reported (*7*, *10*, *11*).

Further studies on the specificity of collagenase from the view point of its action on synthetic substrate will be reported in subsequent publications.

SUMMARY

1. Poly-(L-prolyl-L-leucyl-glycyl) synthesized as collagen model was almost completely digested with purified collagenase to prolylleucylglycylprolylleucine, glycylprolylleucine and glycylprolylleucylglycine at the ratio of 0.8, 19 and 1.0.

2. Glycyl-L-prolyl-L-leucyl-glycyl-L-proline amide synthesized as a possible simplest substrate for collagenase was easily hydrolyzed to glycylprolylleucine and glycylproline amide with the enzyme.

3. From the results obtained above, the specificity of collagenase was discussed.

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Hemoproteins of Wheat Germ

III. Further Studies on Purification of Peroxidase and Some of Their Properties*

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Some years ago, peroxidase 556 and peroxidase 566 were purified and crystallized from wheat germ by a conventional method involving some drastic procedures such as ethyl acetate treatment and acid treatment (*1*). Since peroxidase 566 was labile against such a drastic treatment, it was obtained only with a small yield.

Tagawa and Shin (*2*, *3*) introduced a milder method avoiding such a drastic treatment and obtained crystalline peroxidase 566 in a larger amount, but peroxidase 556 could not be obtained by their method. They also observed by a handspectroscope that only peroxidase 566 was detectable in wheat germ and little peroxidase 556 was in it. Therefore, they thought that peroxidase 566 was the native peroxidase and that peroxidases 556 was a modified peroxidase derived from the former in the course of the preparation. However, there has been only a little experimental evidence supporting their assumption.

The authors improved the method of Tagawa and Shin and obtained the both peroxidases as crystals and studied some of their properties. The results obtained from the present work were apparently against the assumption that peroxidase 556 was derived from peroxidase 566.

EXPERIMENTALS

Materials and Methods

Wheat Germ—Commercial wheat germ (Canadian wheat—Manitoba) was kindly supplied by the Nisshin flour milling Co. Ltd.

Ion Exchange Resins—Amberlite CG-50, type I was termed "Resin A" and type II was termed "Resin B" in the text. These resins were equilibrated with ammonium phosphate buffers.

Ammonium Phosphate Buffer—Stock buffer solution, 0.1 N ammonium phosphate buffer was prepared by mixing M $NH_4H_2PO_4$ and 0.5 M $(NH_4)_2HPO_4$. The mixing ratio of the solutions was indicated as (x : y), x being the acidic solution and y the basic solution. Thus, a buffer solution indicated as 0.05 N (1 : 3) buffer in the text means the buffer which is made by mixing one part of the acidic solution and three parts of the basic solution, and which is 0.05 N in terms of NH_4^+.

Measurement of Absorption Spectra—Hitachi Recording Spectrophotometer, Type EPS-2 was used for the measurement of the absorption spectra.

Ultracentrifugal Analysis and Diffusion Analysis—Sedimentation measurements were made with Hitachi Analytical Ultracentrifuge, Type UCA-1. Diffusion measurements were carried out with Hitachi Electrophoretic Apparatus, Type HTB-2 by using a cell of Neurath type. $S_{20,w}$ and $D_{20,w}$ were determined by the conventional method (*4*).

Purification of Peroxidases

A) *Isolation of Peroxidase 556 and Peroxidase 566 from Wheat Germ*: *Step 1*: *Extraction and Ammonium Sulfate Precipitation*—Each kg. of commercial wheat germ was suspended in 6 liters of 0.1 M sodium phosphate buffer, pH 6.8 at 5°C. The suspension was adjusted to pH 6.8 with 6 N NaOH and homogenized by a mixer. After an hour, 500 g. of ammonium sulfate per one liter of the buffer for homogenization were added to the homogenate without removal of debris. The precipitate was collected by filtration on a Buchner funnel by the aid of celite.

Step 2: *Fraction with Ammonium Sulfate*—The precipitate obtained above was dissolved in five parts

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of 0.1 *M* sodium phosphate buffer, pH 6.8 and then the solution was made to 30% saturation of ammonium sulfate and kept standing overnight at 5°C. The debris and precipitate were removed first by squeezing through heavy cloth and then by filtration. The clear filtrate obtained was made to 62% saturation of ammonium sulfate. Almost all peroxidases were precipitated. They were collected by filtration and ammonium sulfate solution held in precipitate was drained out by pressing between celite beds.

Step 3: Fractionation of Peroxidase 556 and Peroxidase 566 by Resin A—The precipitate obtained above, was dissolved in 0.02 *N* (1 : 3) buffer and the solution was dialyzed against the same buffer for two days in ice cold. The precipitate formed during dialysis was removed by centrifugation. The dialysate containing both of the peroxidases was passed through Resin A column (4×40 cm.) equilibrated with 0.01 *N* (1 : 3) buffer. Peroxidase 566 was adsorbed on the upper portion of the column, while almost all of the peroxidase 556 passed through. The column was washed with 0.01 *N* (1 : 3) buffer until washings became colorless. Then the resin containing adsorbed peroxidase 566 was suspended in 0.02 *N* (1 : 3) buffer, and was washed several times to remove turbidity by decantation. The pH of the resin suspension was adjusted to 7.0 with 15 *N* NH_4OH, and the suspension was transferred into a glass filter funnel with about 0.5 cm. of thick resin bed equilibrated with 0.02 *N* (1 : 5) buffer and washed with the same buffer. Peroxidase 566 was eluted with 0.30 *N* (1 : 5) buffer. This eluate is the crude peroxidase 566.

The solution passed through the Resin A and the washings contained peroxidase 556. They were dialyzed against running tap water and passed through the Resin A column equilibrated with 0.02 *N* (3 : 1) buffer. After being washed with the same buffer, the resin was suspended in 0.02 *N* (3 : 1) buffer and washed three times to remove turbidity by decantation. The suspension of the washed resin was adjusted to pH 6.8 with 15 *N* NH_4OH. By this treatment a part of peroxidase 556 was eluted from the resin. Peroxidase 556 remaining in the resin was eluted with 0.20 *N* (1 : 5) buffer on a glass filter funnel. The filtrate is the crude peroxidase 556.

B) Purification of Peroxidase 566: Step 1: Fractionation on the Column of Resin B—The crude peroxidase 566 obtained in item A was collected by salting out with 72% saturation of ammonium sulfate, and then it was dissolved in 0.05 *N* (1 : 5) buffer and dialyzed against 0.04 *N* (1 : 5) buffer. It was adsorbed on a Resin B column equilibrated with 0.05 *N* (1 : 5) buffer. When 0.05 *N* (1 : 5) buffer was passed through the column, the crude peroxidase 566 adsorbed was divided into four bands. These bands were named as Fraction IV, Fraction III, Fraction II and Fraction I beginning from the upper fraction (see Table II). Fraction I was suspended in 0.025 *N* (1 : 5) buffer and Fraction II, III and IV were separately suspended in 0.05 *N* (1 : 5) buffer. Each suspension was adjusted to pH 7.0 with 15 *N* NH_4OH and then transferred to a glass filter funnel with a thick resin bed, and eluted with 0.25 *N* (1 : 6) buffer.

Step 2: Chromatography—Among the four fractions obtained above, Fraction III contained the largest amount. It was chromatographed on the Resin B column equilibrated with 0.10 *N* (1 : 6) buffer. This fraction was chromatographed repeatedly.

C) Purification of Peroxidase 556: Step 1: Fractionation on the Column of Resin B—The crude peroxidase 556 obtained in item A was collected by salting out with 72% saturation of ammonium sulfate, and then it was dissolved in 0.04 *N* (1 : 3) buffer and dialyzed against 0.02 *N* (1 : 3) buffer. It was adsorbed on the column of Resin B equilibrated with 0.04 *N* (1 : 3) buffer and then developed with 0.03 *N* (1 : 3) buffer. Thus it was fractionated into five fractions on the resin column. Fraction I was not adsorbed under this condition and passed through the column. The other four fractions were adsorbed on the column and they were termed Fraction V, IV, III and II beginning from the upper fraction Fraction II and III were eluted with 0.03 *N* (1 : 3) buffer. After these two fractions were eluted, the eluent was changed to 0.04 *N* (1 : 3) buffer so that Fraction IV and V could be eluted easily.

Step 2: Chromatography—The effluent of Fraction IV was condensed by salting out with ammonium sulfate and then dialyzed. Then chromatography was carried out on the Resin B column equilibrated with 0.035 *N* (1 : 3) buffer. The main fraction obtained was chromatographed repeatedly.

D) Crystallization: The peroxidase in the effluent obtained by chromatography was collected by salting out with ammonium sulfate. The precipitate was dissolved in a minimum volume of 0.1 *M* sodium phosphate buffer of pH 7.0 and then solid ammonium sulfate was added until the solution became slightly turbid. Crystals were formed within a week.

RESULTS AND DISCUSSION

Purification of Peroxidases—In the present method, particular caution was taken in the purification of peroxidase 566 so that the pH was kept at around 6.8 to 7.2 at all times during preparation, and an adequate salt concentration was kept in the solution of peroxidase 566. Since peroxidase 556 was considerably more stable, it was not necessary to make such caution.

Table I

Comparison of Three Methods for Extraction and Dialysis

Procedures		Peroxidases obtained	
Extraction	Dialysis	Peroxidase 556	Peroxidase 566
0.1 *M* phosphate buffer, pH 6.8	phosphate buffer pH 6.8 to 7.2	+	+
water	water	+	−
0.1 *M* KCl	KCl	+	(+)

(+); Modified form of peroxidase

The authors tried to examine three different conditions for extraction and dialysis in the present method. As shown in Table I, while peroxidase 556 was obtained by all these methods tested, peroxidase 566 was obtained only by the first method which made the use of a phosphate buffer. When water was used for the extraction and dialysis peroxidase 566 could not be obtained, and the one obtained by the method using KCl was a modified peroxidase 566. The results indicate that it is important to keep pH constantly at neutral range and the adequate concentration of salt is necessary to keep peroxidase 566 intact. This was also confirmed by the purified peroxidase 566. Although the purified peroxidase 566 was very stable in 0.1 *M* of phosphate buffer, if it was dialyzed against water until salts concentration in the solution became too low for the stability of the enzyme its absorption spectrum was changed as shown in Fig. 1. Comparison with the absorption spectrum of the native peroxidase 566 (Fig. 4) revealed that the β band in the reduced form became only a shoulder. Modification of peroxidase 566 also occurred in an acidic condition. After keeping it in 0.1 *M* acetate buffer, pH 4.0, for two hours at room temperature, peroxidase 566 was adsorbed on the Resin B equilibrated with 0.02 *N* (2 : 1) buffer and eluted with 0.2 *N* (1 : 5) buffer. As shown in Fig. 1, the absorption spectra was changed notably in the Soret region. Therefore, dialysis of peroxidase 566 was carried out against phosphate buffer at pH 6.8 to 7.2.

In Step 2 item A, the peroxidase solution was made to 30% saturation ammonium sulfate, and the solution was kept for one full day in a cold room. The full day standing did help coagulation of contaminating proteins especially of a large amount of particles which were too small to be filtered off on paper. In the method of Tagawa and Shin (*3*), these particles were precipitated during dialysis against water. However, during the dialysis it was sometimes observed the formation of a thin film of these coagulated particles which prevented further dialysis. Moreover as indicated previously, peroxidase 566 was unstable under such highly dilute solution. Therefore, the simple overnight standing was superior for the removal of these contaminating particles.

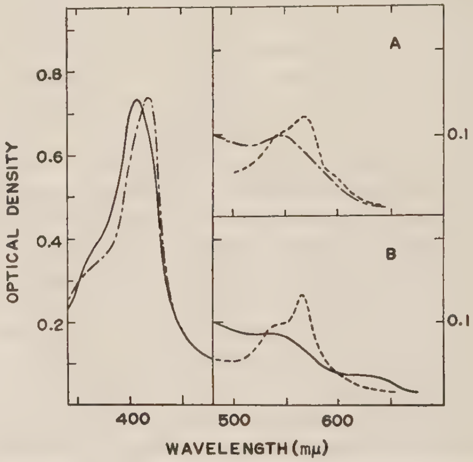

Fig. 1. Absorption spectra of peroxidase 566 modified by dialysis and acid-resin treatment in 0.1 *M* phosphate buffer, pH 7.0.

A: visible region spectra by dialysis

B: visible region spectra by acid-resin treatment

—·—·— oxidized form by dialysis

———— oxidized form by acid-resin treatment

- - - - - reduced form

In Step 1 items B and C, several fractions were obtained by resin treatments. As

TABLE II

Fractionation of Peroxidases by Amberlite CG-50 from 10 kg. of Wheat Germ

Peroxidase 556			Peroxidase 566		
Fractions	Amount (μ moles)	R. Z. (E_{400}/E_{278})	Fractions	Amount (μ moles)	R.Z. (E_{420}/E_{278})
I	2.4	0.11	I	1.5	0.47
II	1.9	0.98	II	1.9	0.25
III	1.1	0.58	III	3.3	0.31
IV	6.7	1.56	IV	1.1	0.16
V	4.7	2.32			

described in Table II, the crude peroxidase 566 preparation was divided into four fractions on a resin column. These fractions appeared to correspond in every way to the four fractions obtained by chromatography in the method of Tagawa and Shin. The absorption spectra of these fractions were shown in Fig. 2. There was an absorption peak at 640 mμ in each of the Fractions I, II and IV. Since the modified peroxidase 566 obtained by acid resin treatment had an absorption peak at 640 mμ, it seems that it is due to the modified peroxidase 566 rather than the 556 type peroxidase. Fraction III obtained was in the largest amount and its absorption spectrum indicated, in respect to an peak at 640 mμ, the presence of a small amount of the modified peroxidase 566. Therefore, it was further purified by chromatography on a Resin B column. The chromatography was carried out three times succes-sively.

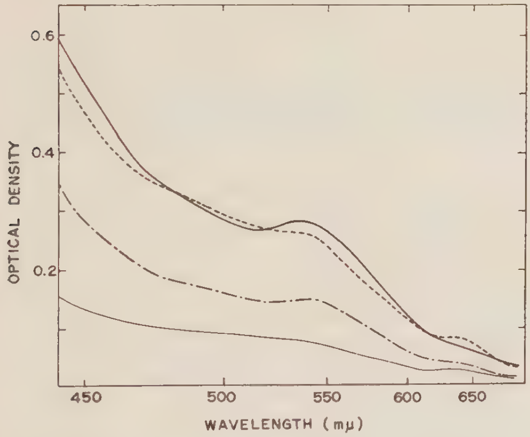

FIG. 2. Absorption spectra of the four fractions obtained by fractionation of the Resin B, Step 1 item B in the text.

—·—·— Fraction I ---- Fraction II

——— Fraction III ——— Fraction IV

In the extraction of peroxidase 556, there were small differences in the absorption spectra between the fractions of the chromatography step. Since Fraction IV was present in the largest amount, it was further purified by chromatography. The chromatography was carried out twice.

By employing the present method, about 4 μmoles of peroxidase 556 and about 2 μmoles peroxidase 566 were obtained as crystals from 10 kg. of wheat germ. 'Reinheit Zahl' was 3.5 for peroxidase 556 and 2.6 for peroxidase 566. These values indicated that these peroxidases seemed to have the highest purity in wheat peroxidases thus far obtained.

Properties of the Peroxidases

Absorption Spectra of Peroxidases—The absorption spectra of peroxidase 556 and peroxidase 566 are shown in Fig. 3 and 4, respectively, and the position of their absorption maxima were summarized in Table III.

The absorption spectrum of peroxidase 566 resembles that of cytochrome b. Consideration of only this point would lead to the idea that peroxidase 566 may be included in the category of b-cytochromes instead of peroxidases. However, cytochrome b in wheat germ mitochondria was insoluble in water and could be solubilized with cholate. wheras the peroxidase 566 was soluble in buffer solution.

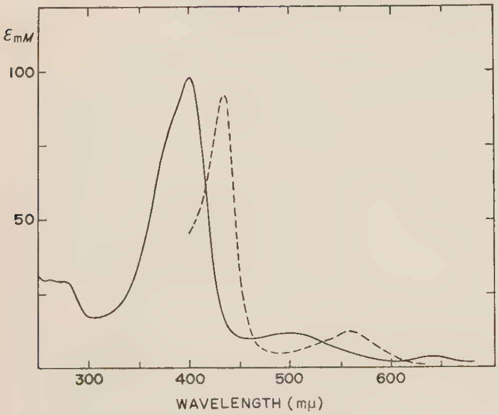

Fig. 3. Absorption spectra of peroxidase 556 in 0.1 *M* sodium phosphate buffer, pH 7.0.
—— oxidized form, - - - - reduced form

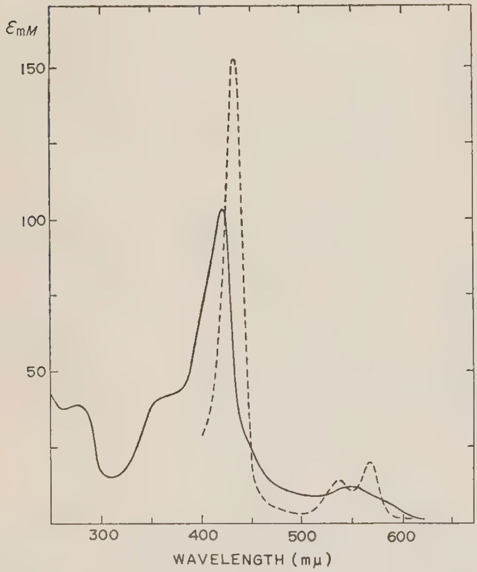

Fig. 4. Absorption spectra of peroxidase 566 in 0.1 *M* sodium phosphate buffer, pH 7.0.
—— oxidized form, - - - - reduced form

In Fig. 1, it is shown that the absorption spectrum of peroxidase 566 was changed by the dialysis against water and the acid-resin treatment. The apparently modified peroxidase 566 having similar absorption spectra was sometimes found in the sample obtained by chromatography such as Fractions I, II and IV (Fig. 5). When each of these apparently modified 566-peroxidases was compared with the native peroxidase 566 in absorption spectra, its Soret band shifts toward

Table III
Absorption Maxima and Extinction Coefficients of Wheat Peroxidases

Peroxidase 556

Oxidized form		Reduced form		Pyridine hemochromogen
Maxima (mμ)	ε m*M*	Maxima (mμ)	ε m*M*	Maxima (mμ)
278	28.1			
400	98.5	435	92.6	420
500	11.5	(520)		480
640	3.1	556	12.0	526 557

Peroxidase 566

Oxidized form		Reduced form		Pyridine hemochromogen
Maxima (mμ)	ε m*M*	Maxima (mμ)	ε m*M*	Maxima (mμ)
278	40.2			
(370)	42.6			
420	105.2	433	155.5	420
545	12.0	536	14.8	480
		566	20.1	526
				557

Calculation of each εm*M* was based on the extinction coefficient, ε m*M*=34.2 for pyridine hemochromogen of protoheme at 557 mμ.

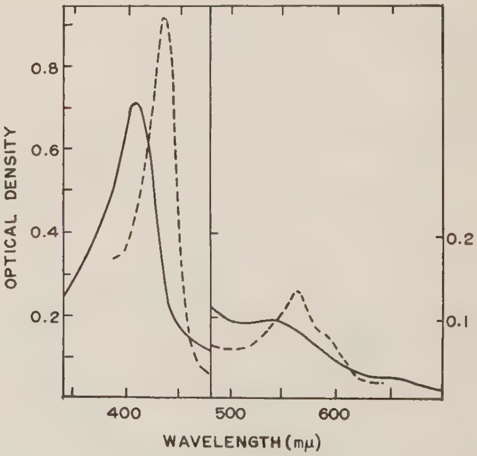

Fig. 5. Absorption spectra of the modified peroxidase 566 in 0.1 *M* phosphate buffer, pH 7.0 (see Table IV).
—— Oxidized form, - - - - reduced form

TABLE IV

Absorption Maxima of the Modified 566-Peroxidases

Peroxidase preparations	Oxidized form (mμ)			Reduced form (mμ)		
preparation in Fig. 5	410	540	640	435	566	(590)
preparation made by dialysis	420	540	(640)	435	566	(590)
preparation made by acid-resin treatment	408	535	640	435	(538)	566
preparation by the method of (*1*)	417	540		433	(538)	566

TABLE V

The Classification of Peroxidases by Morita's Method

Groups	Peroxidases	Absorption maxima (mμ)						Ref.
		Oxidized form			Reduced form			
A	Horseradish peroxidase II	402	500	640	—	558	(594.5)	(*6*)
	Broad bean peroxidase a	404	500	643	439	562		(*7*)
	Japanese radish peroxidase a	405	498	640	439	561		(*8*)
	Peroxidase 556	400	500	640	435	(520)	556	
B	Sweet poteto peroxidase b	418	530	628				(*9*)
	Broad bean peroxidase b	414	530	(640)	428	534	563	(*7*)
C	Horseradish peroxidase I	—	548	583				(*6*)
	Japanese radish peroxidase c	419	540		425	563		(*8*)
	Peroxidase 566	417	540		433	(538)	566	(*1*)

shorter wave length, and its absorptions at 500 mμ and 640 mμ increase in the oxidized forms, and its β band becomes obscure and a shoulder at 590 mμ appears in the reduced forms. Table IV shows absorption maxima of the modified 566-peroxidases.

Many investigators already have isolated peroxidases from various higher plants and studied their absorption spectra. Peroxidases were classified by Morita (*5*) into three groups on the basis of their absorption spectra. Group A has absorption maxima at around 405, 498, 640 mμ in the oxidized form and at 439, 561 mμ in the reduced form. Group B has absorption maxima at around 414, 530 mμ in the oxidized form and at 428, 534, 563 mμ in the reduced form. Group C has absorption maxima at around 419, 540 mμ in the oxidized form and at 425, 563 mμ in the reduced form. Since the absorption spectrm of peroxidase 556 corresponds to that of horseradish peroxidase II, it seems to be a typical peroxidase, and peroxidase 556 may be classified into A group as shown in Table V. It has a maximum at 400 mμ in the Soret region. However, previously it was reported that the peroxidase 556 obtained by Hagihara *et al.* (*1*) had the Soret maximum at 397, and the sample obtained by other method (unpublished) showed it at 403 mμ. It is difficult to explain why there occurred a shift in the Soret region.

In the case of peroxidase 566, it may be classified into B group from the shape of the visible spectrum, because both peroxidase 566 and B group peroxidases have α and β band typical for the absorption spectra of b-cytochromes.

Since, however, peroxidase 566 has a Soret peak at 420 mμ which is a little higher than that in peroxidases of B group of Morita it may be classified rather into his

C group. From a further examination, however, of the absorption spectra of the peroxidases reported by Morita, following points are to be noted. Broad bean peroxidase b, a member of the B group, has an absorption maximum at 640 mμ in the oxidized form and a shoulder at 590 mμ in the reduced form, and Japanese radish peroxidase c, a member of the C group, has no definite β band in the reduced form. Such characteristics appear also in the absorption spectra of the modified peroxidase 566 as shown in Fig. 1 and 5. Therefore, the peroxidases of the B and the C groups described by Morita might possibly be the modified ones derived from the native 566 type peroxidase in each plant. Thus peroxidase 566 appears to be a new type peroxidase according to the Morita's classification.

Prosthetic Group of Wheat Peroxidases—Absorption maxima of pyridine ferrohemochromogen of peroxidase 556 and peroxidase 566 are shown in the Table III. It seems likely that the prosthetic group of the wheat peroxidase is protoheme similar to the one for other peroxidases.

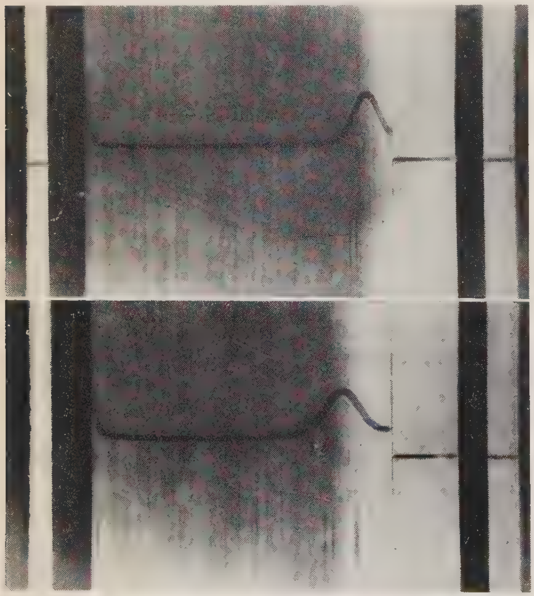

FIG. 6. Sedimentation pattern obtained by peroxidase 556 at 55,200 r.p.m. in 0.1 *M* sodium phosphate buffer, pH 7.0. The concentration of the sample was approximately 0.7 per cent. Pictures shown were taken at 30 and 60 minutes.

Ultracentrifugal and Diffusion Analyses—The homogeneity of the peroxidases obtained was examined by ultracentrifugal analysis as shown in Fig. 6 and 7. Sedimentation constants were calculated at zero enzyme concentration as 3.29×10^{-13} $S_{20,w}$ for peroxidase 556 and 2.68×10^{-13} $S_{20,w}$ for peroxidase 566. The constants were slightly dependent on the protein concentration.

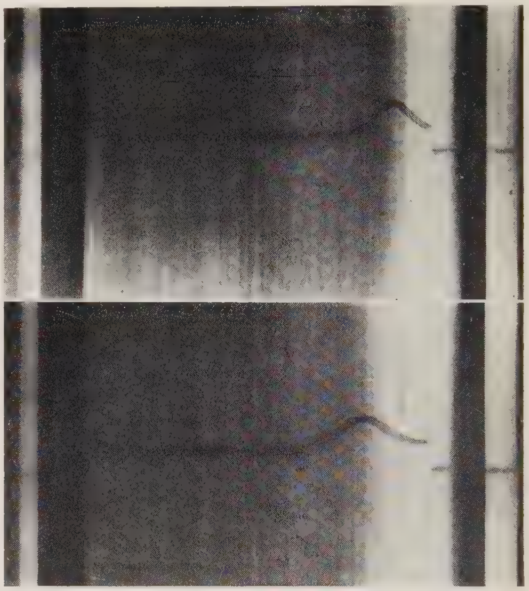

FIG. 7. Sedimentation pattern obtained by peroxidase 566 at 54,600 r.p.m. in 0.1 *M* sodium phosphate buffer, pH 7.0. The concentration of the sample was approximately 0.4 per cent. Pictures shown were taken at 40 and 70 minutes.

Diffusion constants at zero enzyme concentration ($D_{20,w}$) were calculated to be 7.10×10^{-7} cm.^{2}·sec.$^{-1}$ for peroxidase 556 and 7.98×10^{-7} cm.^{2}·sec.$^{-1}$ for peroxidase 566. The constants were also slightly dependent on the protein concentration.

When the partial specific volume was assumed to be 0.75, the molecular weight of peroxidase 556 could be calculated as 45,000 and that of peroxidase 566 was as 32,000 from the above constants.

The physicochemical properties of the peroxidases will be reported elsewhere in more details.

Relationship between Peroxidase 556 and Peroxidase 566—By the present method, both peroxidase 556 and peroxidase 566 were obtained. As described in the report of Tagawa and Shin, under handspectroscope peroxidase 556 could not be observed in wheat germ but it could be observed after wheat germ was treated with acetone, alcohol and ethylacetate, while peroxidase 566 was easily detected. From these evidences together with the observation that only a little peroxidase 556 was isolated from wheat germ, it was suggested that it was derived from peroxidase 566 through some modification during isolation.

However, by the present method so much peroxidase 556 was obtained that it is unlikely that all peroxidase 556 obtained was derived from peroxidase 566 in the course of the preparation. From the sedimentation and diffusion analysis, it was found that peroxidase 556 had larger molecular weight than peroxidase 566, and peroxidase 556 did not coagulate in 25 per cent saturation of ammonium sulfate solution at pH 3.6, while peroxidase 566 coagulated under that condition. These observations suggest that the two peroxidases differ distinctly from each other in their properties and structure. Therefore, it seems that there are two different peroxidases in wheat germ.

SUMMARY

1. An improved method was devised for purification of peroxidase 556 and peroxidase 566 from wheat germ. These peroxidases were obtained as crystals. Their homogeneity was shown by the migration behavior on the resin and by the ultracentrifugal behavior.

2. Peroxidase 566 was found to be a new type of peroxidases. Its absorption spectrum resembled that of b-cytochromes. Modified forms of peroxidase 566 had the absorption spectra similar to some peroxidases obtained by other investigators.

3. Although the peroxidases had protoheme as their prosthetic group, it was suggested from their molecular weights and other properties that properties of protein portions differ distinctly from each other.

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Purification of Hog Thyroglobulin

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The thyroid gland contains a characteristic iodoprotein, thyroglobulin, which plays an essential role in the elaboration, storage, and secretion of thyroid hormones. Although thyroglobulin has been known for a very long time, comparatively little information has been obtained on the nature of this protein.

In 1948, Derrien, Michel and Roche (*1*) proposed a method for the purification of thyroglobulin after a systematic study on its solubility characteristics. They found that thyroglobulin was precipitated without denaturation within a narrow range of concentrations of neutral salts and stated that the preparation they obtained was both ultracentrifugally and electrophoretically homogeneous (*2*). Since then, their procedure has been widely adopted by a number of other investigators for the purpose of purification of thyroglobulin. However, as was already pointed out by O'Donell *et al.* (*3*) and Edelhoch (*4*), the preparation obtained by this method usually contained small amounts of faster and slower sedimenting components as examined in an ultracentrifuge. Edelhoch (*4*) succeeded in further purification by repeated differential centrifugation, but the method was rather poor in yield and is not suited for a large-scale preparation. Therefore, more fundamental work for the purification of thyroglobulin seems to be necessary before thorough investigations on the properties of thyroglobulin are undertaken.

The present paper describes the results of experiments performed in an attempt to establish a procedure for the purification of hog thyroglobulin. Each step of the purification was followed chiefly by the use of an analytical ultracentrifuge, since thyroglobulin has a characteristic sedimentation coefficient as high as 19 S (*5*).

EXPERIMENTALS

Fresh hog thyroid glands obtained from a slaughter-house were used for the purification of thyroglobulin in this study. The glands were freed from fat and extraneous tissues and, in most cases, frozen over solid carbon dioxide as soon as possible. Extraction was started usually within a few hours, while for a large-scale preparation, the glands were stored below −20°C until extraction was started (for a week at longest).

Ultracentrifugal examination was performed in a Spinco model E ultracentrifuge equipped with a rotor temperature indicating and controlling unit. Prior to ultracentrifugation, the solution to be examined was dialyzed against a phosphate buffer of pH 6.5 and ionic strength 0.2, except in the case of extract where the clarified solution was immediately subjected to the centrifugation after appropriate dilution with 0.9% NaCl. Runs were made at a temperature near 20°C and usually at 50,740 r.p.m. The composition of solutions was determined from the relative areas under the peaks in the sedimentation pattern and corrected for the radial dilution in the ultracentrifuge cell.

Analyses were made for nitrogen by the micro-Kjeldahl method and for phosphorus by the method of Knight and Woody (*6*). Iodine was determined by the modified method* of Gross, Wood and McHargue (*7*) after combustion of the sample in a Schöniger flask (*8*). Protein concentration was determined either by nitrogen analysis, by ultraviolet absorption at 280 mμ measured in a Beckman model DU spectrophotometer or by the Folin method modified according to Lowry *et al.* (*9*).

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* Kondo, Y., Tamura, H. and Ui, N., to be published.

TABLE I

Analyses of Extracts Prepared from Different Thyroid Tissue Preparations

Starting Material	Amount of Extracted Proteins (mg./g. wet tissue)	Composition (%)			$\frac{\text{O. D.}_{260\,m\mu}}{\text{O. D.}_{280\,m\mu}}$	P/N× 100 (g./g.)
		F-Comp.	Thyroglobulin	Slow Comp.		
1. Homogenate						
1st Extraction	83	4	78	18	1.10	3.75
2nd Extraction	14	3	72	25	1.18	6.15
2. Frozen Slices						
1st Extraction	71	3	78	19	1.04	2.77
2nd Extraction	9	3	75	22	1.15	5.16
3. Non-frozen Slices						
1st Extraction	49	3	83	14	1.06	3.80
2nd Extraction	30	3	84	13	1.06	—

RESULTS

Extraction of Thyroglobulin and Composition of the Extracts—In order to determine the most suitable condition to extract thyroglobulin, a fresh thyroid gland was divided into three equivalent portions, and they were treated in three different ways. In the first method, a portion of the gland was frozen, minced, and homogenized in a Waring blendor with cold 0.9% NaCl (pH 6.8). In the second method, the tissue was frozen and cut into slices of approximately 1 mm. in thickness. The frozen slices were then suspended in the saline described above. The extraction from frozen thyroid slices was first introduced by Cavett and Seljeskög (*10*) and later recommended by Derrien *et al.* (*1*) as this method makes it possible to extract the materials present in the follicular colloid of thyroid tissue almost exclusively and thus avoids the extraction of the nucleoprotein present in the tissue cells. The fresh tissue without prior freezing was used in the third method. After being sliced, it was treated in the same manner as in the second method. In all of these cases, 3 ml. of 0.9% NaCl was added to each gram of the tissue and extraction was continued overnight at approximately 1°C. Each extract was then separated from the tissue residue by centrifugation at 10,000 r.p.m. for 10 minutes in a refrigerated centrifuge, and the clear supernatant was submitted to examinations.

In Table I are shown the results obtained in a typical experiment. Extracts obtained after re-extracting the residues for 24 hours were also examined and the results are included in this table.

The total amount of proteins extracted was estimated from the nitrogen content of each extract by assuming that the proteins contained 16.0% nitrogen. The calculated amounts were corrected to the values per gram of the wet tissue and they are listed in the second column of Table I. It is seen that the first method, in which the homogenate was used, was most efficient as might be expected. The extraction from the frozen slices was nearly as efficient, while in the case of non-frozen slices a longer time was required to extract.

When these extracts were submitted to the ultracentrifugal analysis, at least three components were observed (see Fig. 1) in agreement with the finding of previous investigators (*11*). The most predominant among them was a component with a sedimentation coefficient at infinite dilution ($s^{\circ}_{20,w}$) of 19.0 S and corresponded to thyroglobulin. A minor component had $s^{\circ}_{20,w}$ of as high as 28 S, and it was tentatively designated as F-component. Another component with the lowest sedimentation coefficient of about 4 S revealed a rather broad boundary. This slow component was found to be heterogeneous, because separation occurred on prolonged centrifugation.

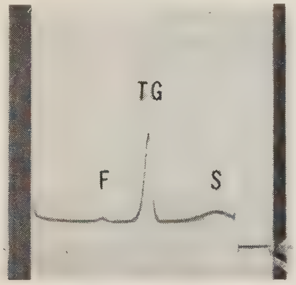

Fig. 1. Sedimentation pattern of thyroid extract (homogenate), 22 minutes after reaching a speed of 52,640 r.p.m. Sedimentation proceeds from right to left. Solvent: 0.9% NaCl (pH 6.8). Concentration: 0.6%.

The composition of the extracts did not differ so much with the method of extraction. The relative concentration of thyroglobulin in the extract from non-frozen slices was only a little higher than that in other extracts. It is also seen in Table I that the first extract was usually richer in thyroglobulin than the second extract.

The fact that the extracts contained nucleic acid might be seen from the last two columns of Table I. Ratio of optical densities at 260 mμ and 280 mμ was definitely higher than the value for purified thyroglobulin (0.62–0.68). Phosphorus/nitrogen ratios of the extracts paralleled with the optical density ratios. It was also indicated that the nucleic acid content in the extract (the first extract) from tissue slices, especially from frozen slices, was lower than that in the homogenate, though the difference was not so great.

From these results, it was considered that the method employing frozen slices was rather excellent. In most of the following experiments, therefore, use was made of the frozen slices for the purpose of extraction.

Although in the above experiment the extraction was continued overnight, it was found, from another experiment, that 3 hours were sufficient for the extraction from frozen slices. Far shorter time would be required in the case of tissue homogenate.

No difference was observed when thyroid glands were extracted with 0.9% NaCl of various pH's between 5.5 and 7.3. Phosphate buffers of pH 6.7 and 7.9 (ionic strength, 0.15) were found to be equally effective.

The relative concentration of thyroglobulin in hog thyroid extracts as revealed by sedimentation analysis varied greatly from gland to gland. The lowest value ever encountered in this laboratory was 11 per cent and the highest was 87 per cent. Most frequently it was 60–80 per cent. The relative concentration of F-component, too, varied markedly with the glands. In an extreme case, F-component was almost absent. Usually its relative concentration was nearly 5 per cent and sometimes reached a value of as high as 10 per cent. The variations in the relative concentrations of thyroglobulin and F-component as well as the variations in the content of these components were found to have something to do with the morphological and other characteristics of the thyroid tissue used (*12*).

Salting-out of Thyroglobulin by Ammonium Sulfate at Low Temperature—In the procedure of Derrien *et al.* (*1*), thyroglobulin is precipitated at 20°C by the addition of ammonium sulfate or equimolar mixture of KH_2PO_4 and K_2HPO_4. However, salting-out at lower temperatures is, no doubt, more desirable in order to avoid proteolysis caused by the enzymes present in the gland (*13*, *14*) and other possible alterations in the protein structure as far as possible. Therefore, solubility of thyroidal proteins in concentrated ammonium sulfate solutions were studied at about 1°C. For this purpose, each 10 ml. of the extract prepared from frozen thyroid slices was placed in a series of centrifuge tubes and mixtures of increasing volumes of 3.50 *M* ammonium sulfate (pH 6.8) and decreasing volumes of distilled water were added to a total volume of 20 ml. The relative concentration of thyroglobulin in the extract used here was 62 per cent and the protein concentration in each tube was constant at 1.1 per cent. After being stood overnight at 1°C, the mixtures were centrifuged in the cold. The protein remaining in the supernatant was analyzed by the ultraviolet absorption at 280 mμ and by the method of Lowry *et al.*

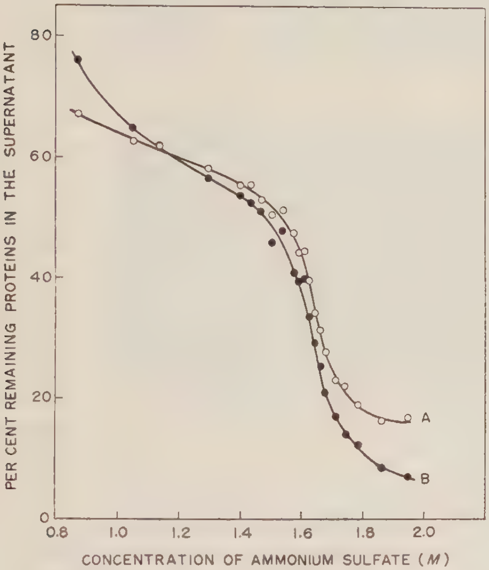

FIG. 2. Salting-out curves for thyroglobulin from hog thyroid extract at 1°C.

Ordinate represents the percent remaining proteins in the supernatant at each concentration of ammonium sulfate. The supernatant at 2.66 *M* was assumed to be free from proteins. Curve A: from optical density measurements at 280 mμ. Curve B: from measurements by the method of Lowry *et al.*

As shown in Fig. 2, the solubility curves obtained by the both methods of analysis fell sharply with an inflection point at 1.65 *M* ammonium sulfate. It was found, from the ultracentrifugal examinations of the supernatants as well as of the precipitates formed, that the protein precipitated in this region corresponded to thyroglobulin, while the slowly sedimenting component present in the original extract precipitated either at lower or higher concentrations of ammonium sulfate. F-component was found to be precipitated together with thyroglobulin.

Taking advantage of the solubility curves, purification of thyroglobulin was undertaken at approximately 1°C as follows (for details, see later section). By the addition of 3.50 *M* ammonium sulfate (pH 6.5) thyroid extract was brought to 1.50 *M* with respect to the concentration of ammonium sulfate. Thyroglobulin was precipitated from the supernatant by 1.80 *M* ammonium sulfate. Then, the precipitate was dissolved in an appropriate volume of 1.55 *M* ammonium sulfate to give an approximately 1% solution, and centrifuged. To the supernatant, 3.50 *M* ammonium sulfate was added until the concentration reached to 1.75 *M* and the formed precipitate was collected. The salting-out between 1.55 *M* and 1.75 *M* ammonium sulfate was repeated three times more.

When the products obtained during these processes were subjected to the analytical ultracentrifugation, thyroglobulin was found to be concentrated gradually. Meanwhile, the slower sedimenting component decreased with repeating of the salting-out. However, as shown in Table II, even the repeatedly purified preparation contained nearly 2 per cent of the slower component. The relative concentration of F-component, on the other hand, was kept almost constant during the processes.

TABLE II

Purification of Thyroglobulin by Ammonium Sulfate at 1°C

Material	Composition (%)		
	F-Comp.	Thyroglobulin	Slow Comp.
Crude Extract	5	72	23
1.50 *M*–1.80 *M* ppt.	5	85	10
1.55 *M*–1.75 *M*			
1st ppt.	6	88	6
2nd ppt.	7	88	5
3rd ppt.	5	92	3
4th ppt.	7	91	2

Even when the salting-out was performed in narrower ranges of ammonium sulfate concentrations than used in the above experiment, the concentration ratio of F-component to thyroglobulin in the products obtained showed nearly the same value. It was indicated that F-component could not be distinguisned from thyroglobulin with respect to solubility in the ammonium sulfate solutions.

Precipitation of thyroglobulin occurred at 20°C at a slightly lower concentration of ammonium sulfate (1.50–1.70 *M*) than at 1°C. F-component again could not be separated from thyroglobulin.

Attempts were made to prepare an ultracentrifugally homogeneous preparation of thyroglobulin by the use of other precipitating agents including equimolar mixture of KH_2PO_4 and K_2HPO_4, but they were unsuccessful.

Further Purification of Thyroglobulin by DEAE-Cellulose Chromatography—In view of the finding that ultracentrifugally homogeneous preparation of thyroglobulin could not be obtained by salting-out methods, attempts were made to purify throglobulin further by means chromatography. On the basis of experiments to be published later (for preliminary report, see (*15*)), the preparation of thyroglobulin obtained by the salting-out with ammonium sulfate was chromatographed on a DEAE-cellulose column (*16*). For this purpose, a solution of thyroglobulin was dialyzed against the phosphate-NaCl buffer of pH 6.5 and ionic strength 0.15 (concentration of NaCl: 0.05 *M*), and applied onto a column which contained DEAE-cellulose (Serva preparation) approximately 70 times the weight (on the dry weight basis) of the preparation. The column was washed with a large volume of the buffer of ionic strength 0.15 in order to remove the small amounts of materials unadsorbed under this condition. Then, the column was eluted with the buffer of higher ionic strength, *i.e.*, 0.40 (concentration of NaCl: 0.30 *M*), at the same pH. Most of the protein applied on the column was recovered in this effluent. When the ionic strength of the eluent was again increased to 2.0, further fraction came off the column. A large part of this fraction was identified by its ultraviolet absorption curve and positive orcinol reaction as ribonucleic acid contaminated in the starting material.

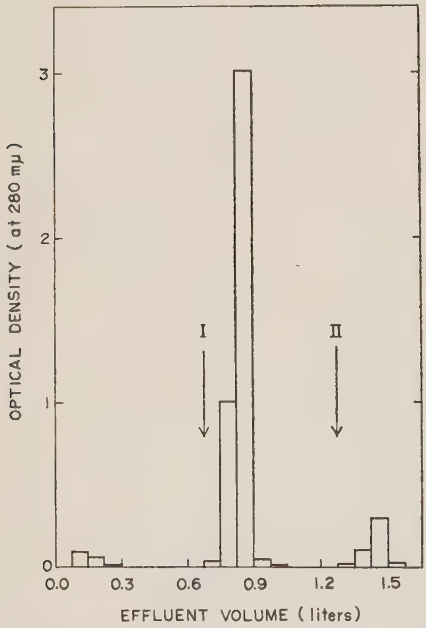

Fig. 3. Chromatography of crude hog thyroglobulin. Column: 4×13 cm. DEAE-cellulose equilibrated with the phosphate-NaCl buffer of pH 6.5 and ionic strength 0.15. Load: 400 mg. Fraction size: 75 ml. Eluents: I, phosphate-NaCl buffer of ionic strength 0.40; II, the same buffer of ionic strength 2.0.

The effluent diagram obtained in a typical experiment is shown in Fig. 3, in which the ordinate represents the optical density of effluents at 280 mμ. It is seen that nearly 80 per cent of the starting material was recovered in the effluent at ionic strength 0.40. Taking into consideration of the fact that nucleic acid has extinction coefficient (at 280 mμ) much higher than that of thyroglobulin, the true recovery would be considerably higher than this value.

In Fig. 4 is shown the ultracentrifugal patterns of the preparations before and after the chromatographic purification. The effluents at ionic strength of 0.15 and 2.0 were also examined in the ultracentrifuge after lyophilization and the patterns are included in this figure (Fig. 4 (D) and (E)). It is seen that the chromatographically purified preparation was quite free from the slower sedimenting component (Fig. 4 (B)). However, F-component was contained in this effluent as much as before the chromatography.

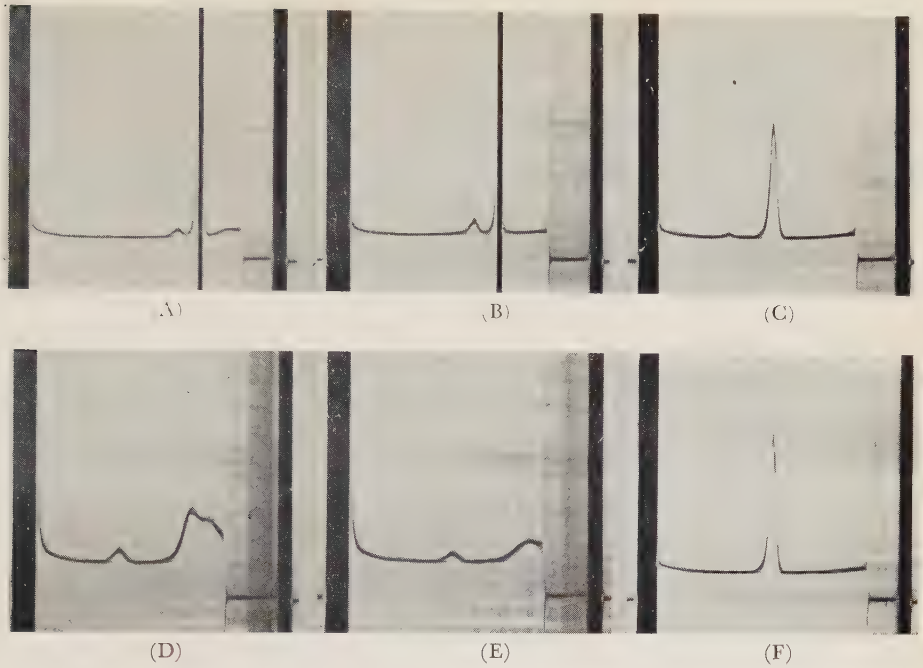

Fig. 4. Sedimentation patterns of hog thyroglobulin preparations in the phosphate buffer of pH 6.5 and ionic strength 0.2. Runs were made at 50,740 r.p.m. except in (F). Sedimentation proceeds from right to left.

(A): Thyroglobulin before chromatography (protein concentration, *ca.* 1%) after 10 minutes.

(B): Effluent at ionic strength 0.40 (protein concentration, 2.3%) after 18 minutes.

(C): The same preparation as (B), but concentration was 0.57%. After 26 minutes.

(D): Effluent at ionic strength 0.15 (protein concentration, 0.7%) after 28 minutes. The fastest small component corresponds to thyroglobulin.

(E): Effluent at ionic strength 2.0 (protein concentration, 0.3%) after 26 minutes. The faster small peak corresponds to thyroglobulin.

(F): Effluent at ionic strength 0.22 (protein concentration, 0.59%) after 27 minutes at 52,640 r.p.m. The absence of F-component is noted.

Separation of 19-S thyroglobulin from F-component could be achieved by changing the ionic strength of the eluent more gradually. When the DEAE-cellulose column was eluted first with a buffer of ionic strength 0.22 instead of increasing directly to 0.40, an ultracentrifugally homogeneous preparation was obtained (see Fig. 4 (F)). Yield was nearly half of the total thyroglobulin applied on the column. F-component, on the other hand, could not be made free from 19–S thyroglobulin, although it was considerably enriched in the effluent at higher ionic strength (0.30-0.40)

It is to be noted that, in the chromatographic purification of thyroglobulin using DEAE-cellulose, the weight ratio of cellulose exchanger to protein was rather critical. When the ratio was higher or lower than that used in the present study, thyroglobulin began to be eluted at higher or lower ionic strength depending upon the ratios*.

The chromatographic purification of thyroglobulin was also found to be successful when the dialyzed thyroid extract was applied on the column without prior precipitation with ammonium sulfate.

For instance, thyroglobulin was partly eluted at ionic strength as low as 0.15 when the weight ratio of cellulose exchanger to protein was 30.

Comparison of the Properties of the Preparations before and after Chromatography—The properties of chromatographically purified preparation were examined and compared with those of the preparation purified by the salting-out method alone. The former preparation was obtained from the effluent at ionic strength 0.40, and contained a small amount of F-component in addition to 19–S thyroglobulin. Re-chromatography was not carried out.

The results are summarized in Table III, which indicates that the chromatography on DEAE-cellulose is highly effective in purifying thyroglobulin.

The chromatographically purified preparation was almost free from phosphorus. Its optical density ratio at 260 mμ and 280 mμ, 0.67, was definitely lower than that of the starting material. These facts indicate that nucleic acid contained in the original preparation was removed successfully by this chromatography. The extinction coefficient of the 1% solution at 280 mμ ($E^{1\%}_{280\,\mathrm{m}\mu}$) markedly decreased after the chromatography.

TABLE III

Properties of Preparations of Hog Thyroglobulin Before and After Chromatography

	Before Chromatography	After Chromatography[1]
N	—	16.0%
I	—	0.43%[2]
P	0.06_1%	<0.005%
O. D.$_{260\,\mathrm{m}\mu}$/O. D.$_{280\,\mathrm{m}\mu}$	0.79	0.67
$E^{1\%}_{280\,\mathrm{m}\mu}$	10.5	9.70
Percent protein hydrolyzed at pH 3.5 (37°C)		
After 2 hours	2.9%	0.4%
After 24 hours	19.3%	0.8%

[1] This preparation was obtained by elution with the buffer of ionic strength 0.40, and contained F-component.

[2] Iodine content was not constant and varied with preparation.

As it was reported that the preparation of thyroglobulin purified by the usual method contained a proteolytic enzyme with an optimum pH of 3–4 (*14*), the degree of contamination was tested using the method similar to that of Kunitz (*17*). To do this, the 1% solutions of these preparations at pH 3.5 were incubated at 37°C for 2 and 24 hours. At the end of the incubation, two volumes of 10% trichloroacetic acid were added to the solutions, and the filtrates were analyzed spectrophotometrically. Judged from the result of this experiment, the chromatographically purified preparation was found to be almost free from the enzyme. The preparation obtained by the salting-out method alone was, on the contrary, hydrolyzed and, after the 2 hours' incubation, 2.9 per cent of the total absorption at 280 mμ became soluble in the trichloroacetic acid solution. After 24 hours' incubation, the value reached to 19.3 per cent.

Preservation of Thyroglobulin Preparations—When the solution of thyroglobulin was lyophilized after dialysis against distilled water, thyroglobulin became partially insoluble in neutral salt solutions. Concentrated thyroglobulin solution in the phosphate buffer of pH 6.5 and ionic strength 0.2, which had been frozen and kept at −20°C for 3 months, showed the presence of a slower component (*ca.* 12 S at 0.5 per cent) when examined in the ultracentrifuge. These preparations were suspected to be partially denatured.

Most satisfactory method for the preservation of thyroglobulin was to store the (wet) precipitate obtained by the addition of ammonium sulfate in a deep-freezer kept below −20°C. Even after 9 months' standing, no change in the ultracentrifugal pattern was observed.

Finally Adopted Method for the Purification of Thyroglobulin—The experiments mentioned above formed the basis of the following method for the preparation of thyroglobulin now adopted in this laboratory. All operations are carried out in a cold room kept at approximately 1°C.

About 100 g. of frozen slices (*ca.* 1 mm. thick) prepared from pre-frozen thyroid glands are suspended in 300 ml. of cold 0.9% NaCl which had been adjusted to pH 6.8.

After being stood for 3 hours with occasional stirring, the mixture is filtered through a double layer of gauze to remove the tissue residue and clarified by centrifugation at 1,000 r. p. m. for 15 minutes in the cold. To the supernatant thus obtained, 3.50 *M* ammonium sulfate (pH 6.8) is added slowly until the concentration becomes 1.50 *M*. After removal of the formed precipitate by centrifugation, ammonium sulfate concentration in the supernatant is brought to 1.80 *M* cautiously. The liquid is stood until bulk of the precipitate is settled down, which usually takes 3 hours or more. Then, the precipitate is collected by centrifugation at 10,000 r. p. m. for 10 minutes. This precipitate is next suspended in 1.55 M ammonium sulfate to give a protein concentration of approximately 1 per cent. Since a well-packed precipitate of thyroglobulin usually contains nearly 90 per cent of the mother liquid, addition of 90 ml. of 1.52 *M* ammonium sulfate per 10 g. of the wet precipitate is a convenient method for this purpose. After stirring the suspension for a few hours, the insoluble matter was separated by centrifugation and discarded. Then, the concentration of ammonium sulfate in the supernatant is raised to 1.75 *M* by the addition of 3.50 *M* ammonium sulfate. The differential salting-out between 1.55 *M* and 1.75 *M* ammonium sulfate is repeated once or twice more. Yield varies from preparation to preparation depending upon the glands used, but is usually 2–4 g. per 100 g. of slices.

For further purification by means of chromatography, the precipitate obtained above is placed in a cellophane tube with a small amount of water and dialyzed against a large quantity of the phosphate-NaCl buffer of pH 6.5 and ionic strength 0.15. DEAE-cellulose is washed with alkali and water and equilibrated with the same buffer. For the chromatography of 1 g. of the sample, 70 g. (dry weight) of the DEAE-cellulose is packed in a glass tube, 4 cm. in diameter and fitted at the bottom with a sintered glass disc. The column reaches the height of nearly 30 cm. The dialyzed thyroglobulin solution is centrifuged to remove the insoluble matter, if present, and applied onto the column. The column is then washed with about 2 liters of the starting buffer of ionic strength 0.15, followed by elution with the buffer of ionic strength 0.40 (concentration of NaCl: 0.30 *M*) at the same pH. The first 250 ml. of the effluent at ionic strength 0.40, roughly corresponding to the hold-up volume of the column, was discarded, and the following 300 ml. portion of the effluent is collected. Thyroglobulin can be precipitated, if not to be re-chromatographed, from this fraction by 1.75 *M* or slightly higher concentration of ammonium sulfate. The wet precipitate obtained by centrifugation is frozen and stored below −20°C until use.

To obtain a thyroglobulin preparation free from F-component, the washed column at ionic strength 0.15 described before should be eluted with the buffer of ionic strength 0.22.

DISCUSSION

The thyroid gland is unique in its histologic structure (*18*). It is composed of a great number of follicles; each of the follicles is lined by a single layer of cells surrounding a cavity. Thyroglobulin is generally considered to be contained in this cavity, so-called colloid, in a state of concentrated solution or gel. In principle, it is, therefore, desirable to extract thyroglobulin without disintegrating the cells surrounding the colloid. Soaking of thyroid slices in cold saline seems to be suitable for this purpose. As even pre-frozen thyroid slices were reported to retain much of the ability to form protein-bound iodine from iodide present in the incubation medium (*19*), most of the thyroid cells in slices are thought to be kept intact during the contact with saline. The present investigaton, in fact, indicated that the extract obtained from frozen or non-frozen thyroid slices was superior to that obtained by homogenization of the tissue; the former contained a lesser amount of nucleic acid than the latter. The frozen slices were thought to be more suitable, since a rather short period was required for the extraction. This comfirms the results of previous investigators (*1*, *10*).

However, it is to be noted that the composition of the extract as revealed by ultracentrifugal analysis varied little with the methods of extraction. In addition, an appreciable amount of nucleic acid was found even in the extract prepared from slices. It remains to be determined whether this fact indicates that the so-called colloid contains nucleic acid (*20*), or thyroid cells are, contrary to our expectation, destroyed appreciably during the process of extraction from slices.

Although the use of tissue homogenate caused the extraction of fairly large amount of nucleic acid, the method is simpler and more effective. As the chromatographic purification method presented in this paper was successful in eliminating the contaminants like nucleic acid, the homogenization method might be no less useful.

In agreement with results of previous investigators (*3*, *4*), the preparation of thyroglobulin purified repeatedly by the fractional precipitation with neutral salts was not homogenous; it contained nucleic acid and othor contaminants. It was true even with the preparation obtained by the low-temperature salting-out method proposed in this paper. However, the latter method perhaps provides the preparation less altered, since the proteolytic enzymes are contained in the crude preparation (*13*, *14*). In additon, the solution of thyroglobulin has a tendency to become turbid when it is stood at room temperature for several hours, and thyroglobulin is considered to be rather heat-labile.

Usefulness of the chromatographic purification by DEAE-cellulose might be emphasized. By employing a simple step-wise elution schedule, removal of the slowly sedimenting component (4 S) was easily achieved. The purity of the preparation was further confirmed by the observations that the product was essentially free from nucleic acid and proteolytic enzymes. The effluent at ionic strength 0.40 contained both 19-S thyroglobulin and F-component (28 S), whereas an ultracentrifugally homogeneous preparation of thyroglobulin was obtained by eluting at a lower ionic strength, *i. e.*, 0.22. In the latter case, however, the recovery was only about 50% of the total 19-S thyroglobulin applied on the column. It is to be noted that thyroglobulin, even if ultracentrifugally homogeneous, is a complex mixture of proteins with different iodine contents and that a fraction with lower iodine content has a tendency to come off the column at a lower ionic strength (*15*). Therefore, the preparation obtained at ionic strength 0.22 would not represent the whole of 19-S thyroglobulin, but contains only fractions with lower iodine contents.

Recently, Ingnar *et al.* (*21*) reported on the chromatographic behavior of sheep thyroglobulin on DEAE-cellulose. They showed, by employing a gradient elution that a fraction in the early portions of the elution curve was ultracentrifugally homogeneous and did not contain the faster sedimenting component. The use of DEAE-cellulose for the chromatographic purification of thyroglobulin was also tried by Schulman and Witebsky (*22*), but they were unable to obtain pure thyroglobulin by chromatography alone. The discrpancy might be due to the difference in elution schedule and, above all, much higher load of proteins they employed.

Of interesting are the presense of F-component in thyloid glands and the possible relationship between this component and thyroglobulin*. The presence of a similar component was reported in human (*23*, *24*), calf (*4*), sheep (*21*), dog (unpublished) and rat (*25*) thyroids without further characterization. F-component was found more or less in hog thyloid extracts, even if a fresh extract was examined in the ultracentrifuge immediately after preparation. The relative concentration of F-component did not depend upon the method of extraction, although it varied markedly with the gland. Furthermore, no increase or decrease in the concentration ratio of F-component to thyroglobulin was observed during the process of purification by ammonium sulfate or during the storage of the partially purified preparation. It was also found that the relative concentration of F-component did not alter with changes in protein concentration or ionic strength of the solutions. Therefore, it seems unlikely that F-component is an artifact produced during the course of the isolation or a polymer formed as a result of monomer-polymer equilibrium. In contrast to the large difference in the sedimentation coefficients, F-component and thyroglobulin have essentially the same solubility characteristics in concentrated salt solutions and migrated in an electric field at the same speed (unpublished data by means of paper electrophoresis). It was also suggested (*15*) that F-component is an iodoprotein like thyroglobulin. Taking into consideration of these findings together with the observation that the content of F-component somewhat depends upon the physiological state of the thyroid gland, it might be reasonable to assume that F-component somehow correlates with the so-called thyroglobulin and plays a physiologically important role in thyroid glands.

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* In this paper, the name of thyroglobulin was given to the thyroidal protein with a sedimentation coefficient of 19 S. As this protein, in fact, contained thyroxine, there remains no doubt in considering that it is principal protein precursor of thyroid hormones. Taking into consideration of the results on F-component (see following discussion), it might be possible that F-componet is also included in the category of "thyroglobulin". However, more, knowledge on this component is required to justify this.

SUMMARY

An improved method for the purification of hog thyroglobulin was presented. The preparation of thyroglobulin, which had been extracted from frozen thyroid slices and repeatedly precipitated by ammonium sulfate at a low temperature, was found to be not homogeneous, and further purification was achieved by chromatography on DEAE-cellulose. Although an ultracentrifugally homogeneous preparation has been obtained by this procedure, the yield was no more than 50 per cent. A part of 19-S thyroglobulin could not be separated from the faster sedimenting component (F-component) of 28 S present in thyroid extracts. The possible relationship between thyroglobulin and F-component was discussed.

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Oxidative N-Demethylation of Aminoazo Compounds by Model System

II. Model System Effected by Ferrous Ion, Ascorbic Acid and Oxygen

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The oxidative N-demethylation of 4-N-dimethylaminoazobenzene (DAB) (*1*), one of the potent hepatocarcinogen in the rat, is more interesting than other metabolic reactions, which include aromatic hydroxylation at 4′ (*2*) and/or 3 position (*3*) and reductive cleavage of the azo bond (*4*), since it is assumed to be concerned most intimately with the formation of the protein-bound aminoazo dyes observed specifically in the target organ (*5*). This metabolic reaction is carried out in liver microsomes and requires both reduced triphosphopyridine nucleotide (TPNH) and oxygen (*6*).

In the previous paper (*7*), the authors reported that the oxidative N-demethylation of DAB could be carried out chemically by each reagent shown as follows; hydrogen peroxide, peroxydisulfate, perbenzoic acid or ferrous ion-oxygen system. As compared with the N-demethylase system located in microsomes of rat liver, the autoxidation system consisting of the iron chelate of ethylenediaminetetraacetic acid (EDTA) and ascorbic acid was rather similar to the above-mentioned biological system of all. This system was reported first as an aromatic hydroxylase model by Udenfriend (*8*), who suggested the possibility that hydrogen peroxide had been formed in the reaction mixture but immediately reacted with some component of the system to yield an actual hydroxylating agent. Acheson (*9*) reported later that the formation of free radicals, which might actually attack the substrate, in the hydroxylating mixture had been confirmed by polymerization experiments. The possibility that a free radical was involved in the aromatic hydroxylation was also shown by Grinstead (*10*), who suggested the participation of hydroxyl or perhydroxyl radical as an active intermediate in the same model system.

On the other hand, a probable mechanism of this system as an oxidative N-demethylase model has not yet been discussed. In the biological system, the oxidative N-demethylation might proceed through a similar chemical pathway postulated in the case of the aromatic hydroxylation, since the former seems to resemble the latter in many respects; *e.g.*, localization in microsomes, requirement of both TPNH and oxygen, and inhibition by common reagents. Therefore, information about the aromatic hydroxylation might also be useful for investigating the oxidative N-demethylation of DAB. As for investigating the mechanism of the oxidative N-demethylation or aromatic hydroxylation, it is desirable to reexamine the above-mentioned autoxidation system; since this system seems to be an important and fundamental one having many similarity to the enzymatic system. The present work intends to investigate the effect of the concentration of each component, *i.e.*, ferrous ion, EDTA, ascorbic acid and oxygen, upon the oxidative N-demethylation of DAB, and thereby to elucidate a role of each component in this system.

EXPERIMENTAL

Material—DAB was repeatedly purified by elution chromatography using alumina (E. Merck AG. for chromatographic adsorption analysis) as an adsorbent. Ferrous ammonium sulfate (E. Merck AG. analytical grade) and ascorbic acid (Daiichi Pure Chemical Co. reagent grade) were made up as a solution with redistilled water just prior to use. Other chemicals were stored as stock solutions. Reactions were carried out by mixing the appropriate amounts of each solution, adding ferrous ion last.

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Procedure of the Reaction—Reactions were carried out for certain periods of time at 35°C in the atmosphere of pure oxygen (Po_2*=785 mmHg). A typical reaction mixture (in 50 ml. of solution) contained: 20 μmoles DAB, 20 μmoles ferrous ion, 40 μmoles EDTA, 400 μmoles ascorbic acid and 2.0 mmoles phosphate buffer (pH 7.2) containing acetone (30 per cent in volume) to dissolve the substrate completely. The reaction was initiated by adding ferrous ion, and terminated by adding large amounts of 0.5% sodium bicarbonate solution. During the course of N-demethylation, the reaction vessel, equipped with a side chamber, was shaken mechanically.

Separation and Determination of N-demethylated Products—The aminoazo dyes including both the substrate (DAB) and the N-demethylated product (MAB)** were extracted three times with each 5 ml. portion of chloroform from the reaction mixture. The combined chloroform extracts were evaporated to dryness under reduced pressure, the residue was dissolved in light petroleum (b.p. 60–80°C) and chromatographed with an alumina column. The light petroleum solution was poured onto the column and the dyes containing both DAB and MAB were developed with benzene-light petroleum mixture. DAB and MAB were eluted with benzene-light petroleum mixture 1 : 4 and 1 : 2, respectively; the effluent containing MAB was evaporated to dryness under reduced pressure. The residue was dissolved in an aliquot of 2*N* HCl-50% ethanol. The amount of MAB was determined by measuring its optical absorbancy at 510 mμ. The absorbancy containing 1 μg. MAB per ml. (thickness of the layer is 1 cm.) corresponds to 0.217.

RESULTS

Effect of Varying Concentration of Ascorbic Acid—The amount of MAB produced by the two hours-reaction increased continuously with increasing concentrations of ascorbic acid (Fig. 1, curve A). When the ratio of MAB formation to ascorbate consumption was plotted against the concentration of ascorbic acid, curve A′ in Fig. 1 was obtained. It indicates that the maximal value which represents the maximal increase of MAB formation is observed at the concentration between 0.8 and 0.4 m*M* (20 and 40 μmoles respectively as shown in Fig. 1). On the other hand, the initial rate of N-demethylation measured in the first 30 minutes-reaction was proportional approximately to the square root of the ascorbate concentration, but it approached to a constant value at higher concentration of ascorbic acid (Fig. 1, Curve B and C).

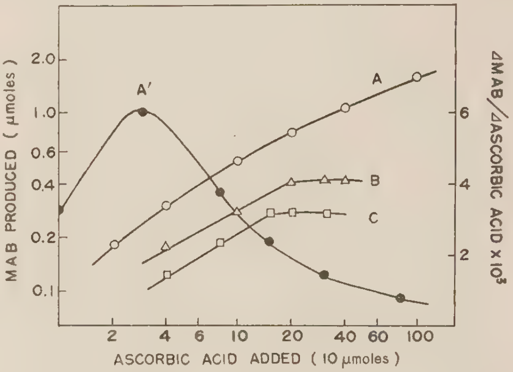

Fig. 1. Effect of varying concentration of ascorbic acid on the N-demethylation of DAB.

Reaction mixtures contained in 50 ml. of 40 m*M* phosphate buffer: curve A & A′; 20 μmoles DAB, 40 μmoles Fe^{2+} and 80 μmoles EDTA, curve B; 20 μmoles DAB, 20 μmoles Fe^{2+} and 40 μmoles EDTA, curve C; 20 μmoles DAB, 5 μmoles Fe^{2+} and 10 μmole EDTA. These mixtures were incubated for 30 min. (curve B & C) or 2 hrs. (curve A & A′) at 35°C.

Effect of Varying Concentration of Ferrous Ion—The rate of MAB production increased steadily with increasing concentrations of ferrous ion. At the concentrations higher than 0.2 m*M*, however, the rate of N-demethylation reached almost a miximal level and remained constant (Table I). In the absence of ferrous ion, the reaction did not occur.

Effect of Ethylenediaminetetraaetic Acid—With increasing concentrations of EDTA, the N-demethylating activity was increased slowly until two moles of EDTA per mole of ferrous ion were added; this amount of EDTA was well above that needed to form the ferrous chelate. Further addition of EDTA reduced the activity of this system (Fig. 2).

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* pressure of oxygen gas

** 4-N-methylaminoazobenzene

TABLE I

Effect of Varying Concentration of Ferrous Ion on the N-Demethylation of DAB

Concentration of Fe^{2+} ion μmoles/50 ml.	MAB found mμmoles/2 hrs.	
0	16.8 ±	4.3[1]
2	438	22
5	644	27
10	958	8
20	996	22
40	1040	17

Reaction mixture contained in 50 ml. of 30% acetone solution; 20.0 μmoles DAB, $2.5 \times [Fe^{2+}]$ μmoles EDTA, 400 μmoles ascorbic acid and 2.0 mmoles phosphate buffer (pH 7.2 ± 0.1). Reaction was carried out at 35°C in the atmosphere of pure oxygen ($Po_2 = 785$mmHg).

1) standard deviation

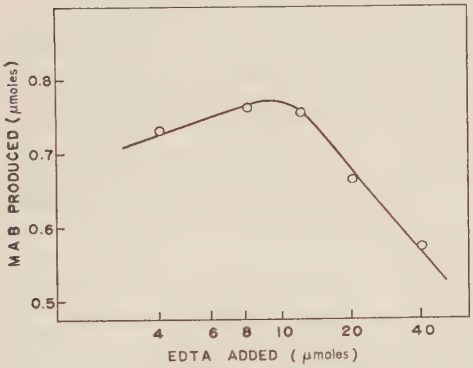

FIG. 2. Effect of varying concentration of EDTA.

Reaction mixture containing 20 μmoles DAB, 40 μmoles Fe^{2+} and 200 μmoles ascorbic acid in 50 ml. of 40 m*M* phosphate buffer (pH 7.2) was incubated for 2hrs. at 35°C in the atmosphere of oxygen ($Po_2 = 785$ mmHg).

Effect of Oxygen—In order to test the necessity of the presence of free oxygen, reaction was carried out in an atmosphere of nitrogen. In this case, the amounts of MAB produced was very small, but not zero, probably because of the difficulty to remove oxygen completely from the reaction mixture (Table II).

TABLE II

Effect of Oxygen on the Amount of MAB Produced in the Reaction System

Atmosphere	MAB found mμmoles/3 hrs.	
Nitrogen	126 ±	22
Oxygen	856	41

Reaction mixture contained in 50 ml. of 30% acetone solution; 15.0 μmoles DAB, 15.0 μmoles Fe^{2+}, 30.0 μmoles EDTA, 300 μmoles ascorbic acid and 2.0 mmoles phosphate buffer (pH 7.3). Reactions were carried out at 35°C in the atmosphere of pure nitrogen or oxygen, pressure of which was 785 mmHg.

Effect of Hydrogen Ion Concentration—With increasing value of pH, there was the considerable increase in the amount of MAB produced as well as in the rate of oxidation of ascorbic acid. At higher pH region above 7.5, ascorbic acid became instable, and the rate of N-demethylation was reduced rapidly (Fig. 3).

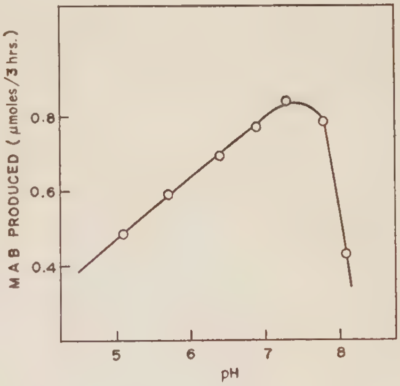

FIG. 3. Effect of hydrogen ion concentration on the N-demethylation of DAB.

Reaction mixture contained in 50 ml. of 40 m*M* phosphate buffer: 15 μmoles DAB, 15 μmoles Fe^{2+}, 30 μmoles EDTA and 300 μmoles ascorbic acid.

T=35°C, $Po_2 = 785$ mmHg.

Effect of Temperature—The amount of MAB produced increased steadily with increasing temperature. At elevated temperature above

40°C, however, the amount of MAB was decreased, presumably as a result of the decomposition of ascorbic acid (Fig. 4).

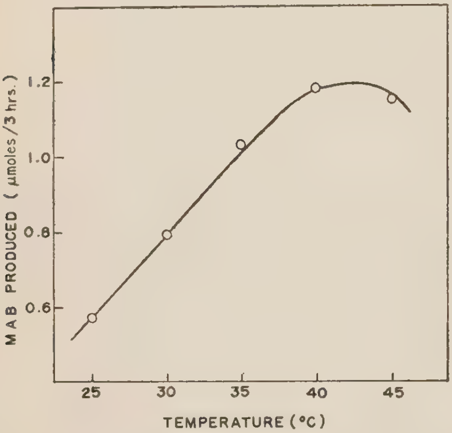

FIG. 4. Effect of temperature on the reaction system.

Reaction mixture contained in 50 ml. of 40 mM phosphate buffer (pH 7.2); 15 μmoles DAB, 15 μmoles Fe^{2+}, 30 μmoles EDTA and 300 μmoles ascorbic acid. P_{O_2}=785 mmHg.

Effect of Metal Ion—Metal ions were essential to catalyze the reaction. The effect of ferric ion on the rate of N-demethylation was nearly equal to that of ferrous ion, whenever ascorbic acid was present in the reaction mixture. Other metal ions were of less or no effect; *e.g.*, cupric and cobaltous ions were about 5 per cent as effective as ferrous ion, but zinc ion was almost ineffective (Table III).

TABLE III

Effect of Metal Ion on the Amount of MAB Produced in the Reaction System

Metal Ion	MAB found mμmoles/2 hrs.		
Fe^{2+}	995	±	20
Fe^{3+}	991		20
Cu^{2+}	51		2
Co^{2+}	50		4
Zn^{2+}	37		10
None	17		4

20.0 μmoles DAB, 20.0 μmoles metal ion, 50.0 μmoles EDTA, 400 moles ascorbic acid and 2.0 mmoles phosphate buffer (pH 7.2) were contained in 50 ml. of the reaction mixture (30% acetone solution). Reaction mixture was shaken at 35°C in the atmosphere of oxygen (P_{O_2}=785 mmHg).

Effect of Reducing Agent—Reduction of ferric ion by some favorable hydrogen donor may be necessary for the N-demethylation. In order to estimate the activity of the reducing agent in the model system, other systems containing either thioglycolic acid or hydroquinone in the place of ascorbic acid were constructed, but no appreciable effects were observed on the N-demethylation of DAB (Table IV).

TABLE IV

Effect of Reducing Agent on the Amount of MAB Produced in the Reaction System

Reducing Agent	MAB found mμmole/2 hrs.		
Ascorbic Acid	959	±	45
Thioglycolic Acid	56		5
Hydroquinone	57		5
None	52		5

Reaction mixtures contained in 50 ml. of 30% acetone solution; 20.0 μmoles DAB, 20.0 μmoles Fe^{2+}ion, 40.0 μmoles EDTA, 400 μmoles reducing agent and 2.0 mmoles phosphate buffer (pH 7.2).

T$^{1)}$=35°C, P_{O_2}=785 mmHg.

1) reaction temperature

DISCUSSION

The simplest system for the oxidative N-demethylation of DAB can be constructed with the mixture of the ferrous chelate of EDTA and oxygen, but the amount of MAB produced is too small to study quantitatively (*7*). When ascorbic acid was added into this reaction mixture, the N-demethylating activity increased pronouncedly; *e.g.*, by adding 5 moles of ascorbic acid per mole of ferrous ion, the amounts of MAB produced was increased about seven-fold as much. On the other hand, the ferric chelate itself could not display any activity to oxidize DAB, even if free oxygen was bubbled vigorously into the reaction mixture.

Ascorbic acid itself has not the activity to N-demethylate DAB, because it can not produce any amount of MAB in the absence of ferrous ion. When ascorbic acid is present in the reaction mixture, the ferric chelate can display its activity to produce MAB. The activity of the model system containing ferric ion in the place of ferrous ion was almost equal to that of the ferrous ion system as observed in the steady state, but showed a lag period in the reaction of the initial state, presumably due to the necessity of the reduction of ferric to ferrous ion (Fig. 5). In the reaction system consisting of the ferrous chelate, ascorbic acid and oxygen, ferrous ion was oxidized rapidly during the course of N-demethylation, especially in the early period, and the continuous decrease in the rate of MAB production followed. In such a case, the addition of more ascorbic acid could help the system become reactive again and resulted in the increase in the amount of MAB produced.

If the reduction of the chelated ferric ion by ascorbic acid is coupled with the oxidative N-demethylation of DAB, the rate of the former reaction will be related to that of the latter. Since there is some evidenec that the rate of the redox reaction may be related to its standard free energy change (*11*), it is probable that the rate of reduction of the chelated ferric ion by ascorbic acid is related to its favorable standard free energy change. In the reaction between the ferric chelate and ascorbic acid, the overall reaction can be represented:

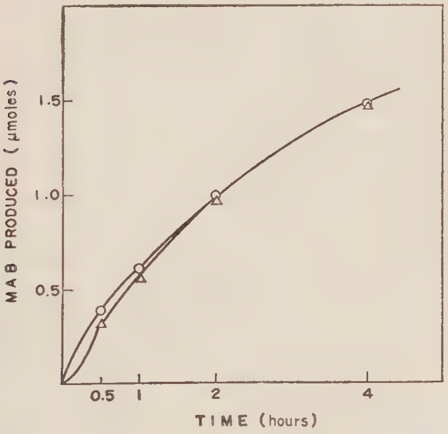

Fig. 5. Effect of the ferrous and ferric ion system on the rate of MAB production.

—○—; the ferrous ion system

—△—; the ferric ion system

Reaction mixture contained 20 μmoles DAB, 40 μmoles Fe^{2+} or Fe^{3+} and 400 μmoles ascorbic acid in 50 ml. of 40 m*M* phosphate buffer (pH 7.2).

$T=35°C$, $Po_2=785$ mmHg.

$$AH_2+2FeY^{-}\longrightarrow A+2FeY^{=}+2H^{+} \qquad \text{(eqn. 1)}$$

where FeY^{-} and $FeY^{=}$ represent the ferric and ferrous chelate of EDTA, and AH_2 and A ascorbic acid and dehydroascorbic acid, respectively. The standard free energy change (ΔF_0) corresponding to the above reaction is given by

$$\begin{aligned}\Delta F_0 &= -2\mathfrak{F}[E_0(FeY^{=}-FeY^{-})-E_0(AH_2-A)]\\ &= -2\mathfrak{F}\Delta E_0 \qquad \text{(eqn. 2)}\end{aligned}$$

where $\mathfrak{F}$ is Faraday constant, $E_0(FeY^{=}-FeY^{-})$ is the standard redox potential of the $FeY^{=}-FeY^{-}$ couple (*12*) and $E_0(AH_2-A)$ that of the AH_2-A couple (*13*). If the rate and ΔF_0 of the above reaction is related, it follows from equation 1 that a similar relation should exist between the rate and ΔE_0 value. When the rate of the ferric chelate-reduction is compared with that of N-demethylation, ΔE_0 value may replace the rate constant of the former reaction. The rate of N-demethylation, as well as the rate of the ferric chelate-reduction, increases with decreasing concentrations of hydrogen ion. At several hydrogen ion concentrations, a plot of log k′ (logarithmic value of apparent N-demethylation rate) against $\Delta E_0'$ is shown in Fig. 6. Its linearity indicates a direct correlation between the rate of N-demethylation and that of reduction of the ferric chelate.

As the reaction proceeds, ascorbic acid disappears rapidly and dehydroascorbic acid or other oxidized intermediate, such as the ascorbate radical, are produced succesively. It is interesting to see that these oxidized products seems to be also effective on the N-demethylation, because MAB is produced in the reaction mixture even when ascorbic acid is almost disappeared. In this connection, Udenfriend (*8*) has shown that dehydroascorbic acid, as well as other compounds

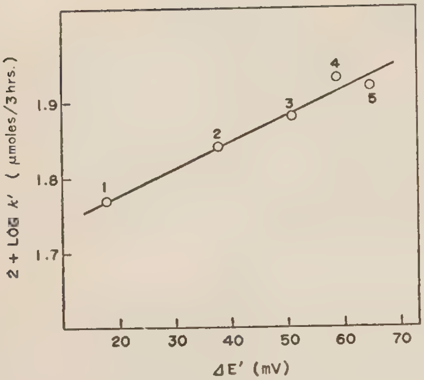

FIG. 6. The apparent rate of N-demethylation plotted against the standard redox potential difference for the reduction of the ferric chelate.

1, pH 5.7; 2, pH 6.4; 3, pH 6.9; 4, pH 7.3; 5, pH 7.6. The reactions were carried out in the same condition as shown in Fig. 3.

having an ene-diol or a diketo structure, is able to accelerate the aromatic hydroxylation though it can replace ascorbic acid only below pH 6.5. When a reducing agent having neither an end-diol nor a diketo structure, such as thioglycolic acid or hydroquinone, was utilized in the place of ascorbic acid, the system could exhibit no activity to produce MAB. Neverthless, there remains considerable doubt whether the effective reducing agents on the N-demethylation are those having an ene-diol or a diketo structure or those having other common properties.

Another important component of the N-demethylase model system is oxygen. The presence of oxygen in an atmosphere seems necessary for the system to catalyze the reaction. In the aromatic hydroxylation, it is shown that free oxygen is incorporated into the aromatic ring of the substrate. Mason (*14-15*) has shown that crystalline horse-radish peroxidase can act as an aromatic hydroxylase in the presence of dihydroxyfumaric acid and that oxygen which is incorporated into the substrate as hydroxyl group is found to arise from the atmosphere. In the oxidative N-demethylation, the possibility may be suggested that oxygen is incorporated into the most reactive site of DAB, presumably nitrogen atom at dimethylamino group (*16*, *17*), to from labile intermediate, which in turn decompose to yield MAB and formaldehyde.

SUMMARY

In the reaction system containing the ferrous chelate of EDTA and oxygen, DAB can be oxidatively N-demethylated to yield MAB. Ascorbic acid, added into the above reaction mixture, can increase the rate of N-demethylation, but can display no activity in the absence of the ferrous chelate. At several hydrogen ion concentrations, the reaction rates increase as the standard redox potential difference between the ferric-ferrous chelate couple and the ascorbate-dehydroascorbate couple increases. Ascorbic acid itself may behave like a cofactor which reactivate the system. Though this model system required the presence of free oxygen, it was not clear whether oxygen derived from the atmosphere was actually incorporated into the reaction intermediate or not.

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Studies on the Protein Synthesis in Silkglands

II. Effects of Inhibitors on the Transfer of Radioactivity and Role of Lipid Fraction in the Protein Synthesis*

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The authors revealed in the previous paper (*1*) that the radioactive substances existing in prelabelled cell debri (CD) were transferred enzymatically into particulate protein in the silkworms' posterior silkglands.

In the present communication the studies of the effects of some inhibitors on the transfer of radioactivity from prelabelled CD to particulate protein are reported. The role of lipid fraction in the protein synthesis has also been studied and discussed.

EXPERIMENTAL

Materials and methods were essentially the same described in detail in the preceding paper (*1*).

Animals, a) domestic silkworms, *Bombyx mori* L., at the 6th to 7th day of the 5th instar larvae; b) albino rats weighing approximately 100 g. were used.

Radioactive amino acids, glycine-2-C^{14} and glycine-1-C^{14} were purchased from Daiichi Pure Chemicals & Co., Tokyo. C^{14}-amino acids mixture (algal protein hydrolysate) was kindly supplied from the Institute of Applied Microbiology, The University of Tokyo.

Ascites-hepatoma cells of the strain AH 130 were transplanted intraperitoneally to albino rats and the animals were sacrificed after 7–10 days of inoculation. Both ascitic fluid and ascites hepatoma cells were collected. Regenerating liver was obtained from rats 48 hours after partial hepatectomy.

Silkglands homogenates and cellular fractions were prepared following the procedures of Shimura *et al.* (*2*). For the preparation of prelabelled CD (C^{14}-CD), minced silkglands were labelled with glycine-2-C^{14} at 37°C for 15 minutes, homogenized in a Waring blendor, centrifuged at $700\times g$ for 10 minutes. Pellets were collected and treated as previously described (*1*).

Incubation procedures were essentially the same as described previously (*1*) and are indicated in the legends of the tables and figures.

By the method of Huggins and Cohn (*3*), lipid, phosphatido-peptide and protein were extracted and plated on aluminum planchettes and dried. The radioactivity was counted in a windowless Q-gas flow counter, corrected for self-absorption and expressed as specific activity with reference weight of 10 mg. (*4*).

RESULTS AND DISCUSSION

Effects of Inhibitors on the Transfer of Radioactivity from Prelabelled CD to Particulate Fractions—The results of the inhibition study on the transfer reaction are given in Table I. Some inhibitors were added directly in the post-incubations, while in several cases C^{14}-CD were preliminary treated with nucleases *etc.* ("pretreatment") and added to the reaction mixture in the succeeding incubations ("post-incubation").

A cell free preparation of $RL+E_1+E_2+E_3$ was used as the reaction system in the postincubation.

All the pretreatments and postincubations were run at 37°C for 30 minutes, with constant shaking. Pretreated CD was washed thoroughly

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Abbreviations: ATP, adenosine triphosphate; GTP, guanosine triphosphate; tris, tris (hydroxymethyl) aminomethane; RNase, ribonuclease; DNase, deoxyribonuclease; DOC, sodium deoxycholate; PCA, perchloric acid; TCA, trichloroacetic acid; CD, cell debris; RL, large particles; E_1, small particles; E_2 and E_3, enzyme fractions.

TABLE I

Effect of Inhibitors on the Transfer of Radioactivity from C^{14}-CD to Particulate and Supernate Fractions

Pretreatment of prelabelled CD[1]	Added inhibitors in postincubations[2]	Radioactivity transferred to			
		RL fraction			Supernate (E_{2+3})
		Lipid	Phosphatido-peptide	Protein	Protein
		c.p.m./mg.	c.p.m./mg.	c.p.m./mg.	c.p.m./mg.
None	—	—	0.7	169 (100%)	11
RNase (100μg./ml.)	—	0	0	66 (39)	7
DNase (100μg./ml.)	—	0	0	71 (42)	4
Deionized water	—	0	1.4	25 (15)	3
Deoxycholate (0.5%)	—	0.7	0.3	37 (22)	4
—	Chloramphenicol (10^{-4} *M*)	0.9	0	197 (117)	10
—	Nitrogen mustard (10^{-5} *M*)	7.3	0	250 (148)	11
—	2, 4-Dinitrophenol (10^{-4} *M*)	—	0	209 (123)	9

1) Prelabelled CD were incubated with the inhibitors in buffered 0.4 *M* sucrose (containing Mg^{2+}) (*2*) except the case of pretreatment with deionized water, in a final volume of 2.0 ml., pH 7.4–7.6. After the treatments, these inhibitors were eliminated by washing several times with buffered 0.4 *M* sucrose (containing Mg^{2+}).

2) Incubation systems consisted of: 16 μmoles of ATP, 0.5 μmole of GTP, 20 μmoles of phosphocreatine, 1.0 mg. of crude creatine kinase, 8 μmoles of $MgCl_2$, 120 μmoles of KCl, 100 μmoles of tris-HCl (pH 8.0), 800 μmoles of sucrose, 19 amino acids mixture less glycine*, RL (9.8 mg. of protein), E_1(9.4 mg. of protein), E_2(7.8 mg. of protein), E_3(3.6 mg. of protein) and C^{14}-CD (565 c.p.m./mg.) as the C^{14}-source, total counts of 21,000 c.p.m./vessel; in a final volume of 2.0 ml., pH 7.4–7.6.

with buffered 0.4 *M* sucrose (*2*) to eliminate those inhibitors in the postincubations.

As can be seen from Table I, the addition of chloramphenicol (10^{-4}*M*), nitrogen mustard (10^{-5}*M*) and 2, 4-dinitrophenol (10^{-4}*M*) caused no inhibition, but had a slight stimulatory effect on the transfer reaction of radioactivity from C^{14}-CD to particulate protein.

By RNase and DNase treatments, however, the radioactivity transferred to RL-protein was reduced considerably.

Above all, washing with deionized water and DOC-treatments showed a marked inhibitory effect on the transfer reaction.

Table II shows a further analysis of the treated CD and its correlation with the transfer of radioactivity from treated C^{14}-CD to particulate protein.

By RNase treatment about 30 per cent of the radioactivity was lost from CD and the transferring activity was also suppressed, whereas by DNase treatment it was noted that there was a little less inhibition than had occurred in RNase treatment.

These facts indicate the possible involvement of nucleic acids in the transfer reaction.

When the prelabelled CD were incubated in deionized water, however, a large amount of radioactive substances including protein were released and lost. By DOC-treatment, on the other hand, the transfer was markedly suppressed although its radioactivity was almost completely preserved.

Consequently, it might be thought that some lipoidal materials lost by DOC-treatment made an important contribution as a transferring factor to the transferring reaction.

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* personal communication of Dr. K. Shimura (*1*).

Table II

Effect of Pretreatments on Labelled CD

Treatment	Specific activity of labelled CD after the treatment	Protein-bound radioactivity of treated CD	Radioactivity transferred to RL protein (from Table I)
	c.p.m./mg.	c.p.m./mg.	%
None	565 (100%)	2133	100
RNase (100 μg./ml)	398 (70.5)	2201	39
DNase (100 μg./ml)	499 (88.3)	2637	42
Deionized water	366 (64.8)	1815	15
DOC (0.5%)	563 (99.6)	2221	22

Comparing the protein-bound radioactivity of all the pretreated CD, there was no appreciable difference between them except the case of treatment with deionized water. Thus it is suggested that the "protein fraction" of C^{14}-CD essentially is the protein which is inherent with CD. Accordingly the protein-bound radioactivity in the CD fraction might not be associated with the transfer of radioactivity, but with some peptidelike substances as related elsewhere.*

Fig. 1. shows a time course of incorporation *in vitro* of glycine-2-C^{14} into particulate and supernate protein in minced silkglands. And Fig. 2, two phase incubation: for the first 10 minutes with C^{14}-glycine followed by the subsequent incubation with C^{12}-glycine.

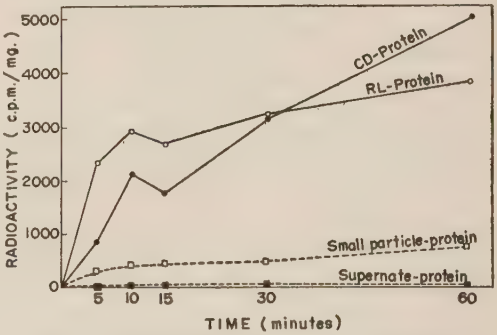

Fig. 1. Incorporation *in vitro* of glycine-2-C^{14} into particulate protein in minced silkgland.

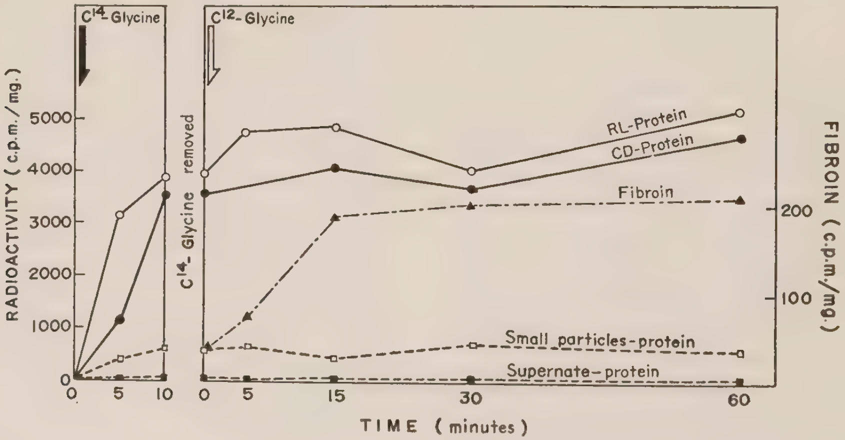

Fig. 2. Incorporation *in vitro* of glycine-2-C^{14} into particulate protein in minced silkgland by an interrupted incubation.

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* will be discussed in the succeeding paper.

As for the time courses it seems difficult to find out a particular relation between CD-protein and particulate or supernate protein.

From these results, it can be concluded therefore that CD-protein *per se* is not transferred to particulate or supernate protein.

While in bacterium it was reported that the cell wall peptide and cytoplasmic protein were synthesized independently (*7*), the possibility of independent synthesis of CD and particulate protein in the posterior silkgland might likewise be supposed.

Role of Lipid Fractions in the Protein Synthesis in Silkglands—As refered in the previous paper (*1*), there have been some doubts on the problem that silk protein is synthesized through the process of "conventional" protein synthesis proposed by Hoagland *et al.* (*8*). And the possibility of another pathway in the protein synthesis in silkworms' silkglands was suggested by Shimura *et al.* (*9*).

In the recent time, the role of lipid fraction in the protein synthesis has also been introduced. Hendler (*10*, *11*) indicated the direct involvement of lipids in the protein synthesis on hen oviduct tissue, while Huggins and Cohn (*3*) regarded phosphatido-peptide as a precursor of protein. For example, according to Hendler (*11*), amino acids were first of all rapidly incorporated into the lipid fraction of CD, and would make an intermediate of protein.

In fact, as can be seen from Figs. 3 and 4, the slices of normal rat liver and regenerating liver showed a relatively high incorporation of C^{14}-amino acids into the lipid fraction compared with that of the protein fraction, and that by homogenation of the tissues, incorporation of C^{14}-amino acids into lipid fraction was markedly diminished as well as that of the protein fraction. These facts lead us to an experiment on the possible involvement of the lipid fraction in the protein synthesis, with the participation of the cell structures.

In the silkworm's posterior silkgland, however, the incorporation *in vitro* C^{14}-glycine into lipid and phosphatido-peptide fraction was by far at a lower rate than that of the protein (Fig. 5). Also the same tendency was recognized on the occasion of C^{14}-amino acids mixture used as the C^{14}-source (Table III).

As to the distribution of radioactivity in the cellular fractions, either in experiments *in vivo* (*1*) or *in vitro* (Table IV), similar results were found as indicated above.

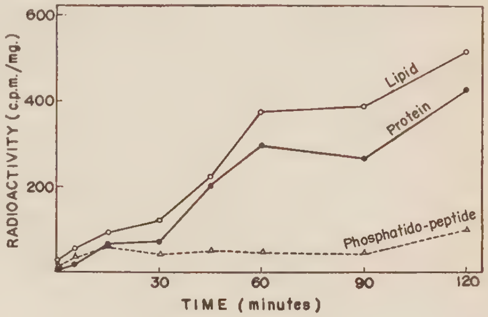

FIG. 3. Incorporation *in vitro* of C^{14}-all-labelled amino acids into lipid, phosphatido-peptide and protein fraction of normal rat liver slices.

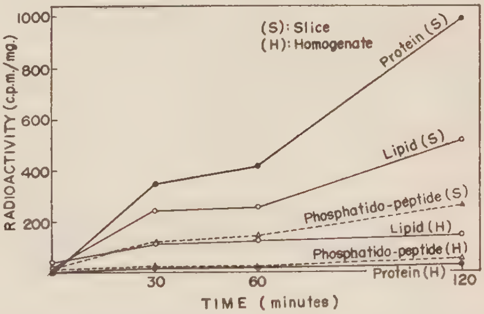

FIG. 4. Incorporationn *in vitro* of C^{14}-all-labelled amino acids into regenerating rat liver slices and homogenates

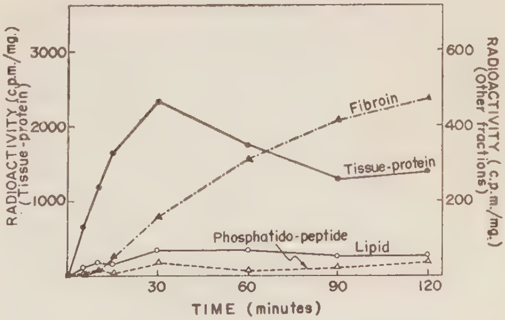

FIG. 5. Incorporation *in vitro* of glycine-1-C^{14} into the minced posterior silkglands.

Table III

In Vivo Incorporation of C^{14}-Amino Acids into Lipid, Phosphatido-peptide and Protein Fraction of the Posterior Silkgland during 30 Minutes

Fractions	Distribution of radioactivity			
	Lipid	Phosphatido-peptide	Protein	Fibroin
Radioactivity incorporated	c.p.m./mg. 43	60	2390	10

1) Silkworms were injected 2 μc per capita of C^{14}-amino acid mixture, and 30 minutes later the posterior silkglands were collected.

2) Fibroin was separated from silkglands by TCA-method (*6*).

Table IV

Incorporation in Vitro of Glycine-2-C^{14} into Cellular Fractions of Minced Silkglands

Incubation time (min.)	Cell debris (CD)			Large particles (RL)			Small particles	Supernates
	Lipid	Phosphatido-peptide	Protein	Lipid	Phosphatido-peptide	Protein	Protein	Protein
			c.p.m./mg.			c.p.m./mg.	c.p.m./mg.	c.p.m./mg.
5	7	13	862	7	9	2321	266	18
10	22	16	2133	10	12	2867	400	27
15	14	10	1749	16	9	2663	414	27
30	19	12	3195	7	11	3200	460	19
60	33	30	5114	14	13	3828	744	46

1) Incubation system was the same as those described in the legend of Fig. 1.

2) Small particles: 105,000×*g* precipitates.

No initial marked uptake into lipid fractions as indicated by Hendler (*10*), was observed. After all, no positive evidence that provides the lipids as the precursor of protein was obtained in this series of experiments.

In the rat ascites hepatoma cells, the same situation was also observed (Fig. 6). At any rate, it is of interest to indicate that only a small incorporation into the lipid fraction was observed in such tissues, showing an excellent protein synthesis as silkworms' posterior silkglands and rat ascites tumor cells. However as pointed out by one of the authors (Miura) a correlation was found between the incorporation rate of amino acids into protein and into lipid fraction which was observed in the other experiment (*12*).

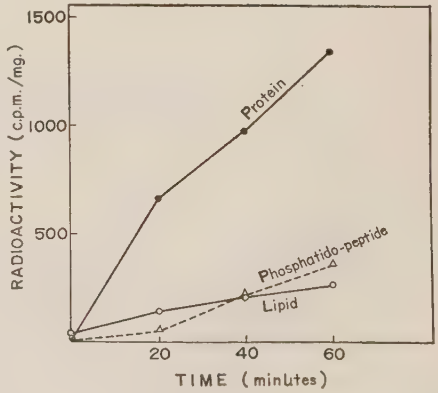

Fig. 6. Incorporation *in vitro* of C^{14}-all-labelled amino acids into rat ascites hepatoma cells.

As regards the distribution of radioactivity which was incorporated into the lipid fraction of the silkgland, it was reported that the bulk of the radioactivity was detected in the protein moiety of proteolipids (*13*). The authors also obtained similar results, whereas Haining, Fukui and Axelrod (*14*) indicated on rat liver that a greater part of the radioactivity incorporated into the lipid fraction was found in nonphosphatides such as triglyceride, sterol and sterol ester. These were exceedingly stable chemically and metabolically, apart from their possible participation in the protein synthesis.

On the other hand, the authors have developed studies on the transfer of radioactivity from C^{14}-prelabelled silkgland CD to the particulate protein, and a relatively efficient incorporation into the particulate protein was recognized. When the CD were preliminary treated by DOC, as already mentioned, the transfer of radioactivity was markedly suppressed although their radioactivity was almost completely preserved.

Hence it might be supposed that the lipid fraction is not a direct precursor of protein in silkglands, but mediates the transfer reaction as a transferring factor.

An attempt to elucidate the transferring substances which may exist in CD and transferred enzymatically to the particulate protein will be reported elsewhere.

SUMMARY

Studies on the effects of inhibitors upon the transfer of radioactivity from prelabelled silkgland cell debri to particulate protein have been developed. The role of lipid fraction in the protein synthesis has also been studied and discussed.

1. At the transfer of radioactivity from prelabelled cell debris to particulate protein, RNA, DNA and deoxycholate-soluble lipoidal materials were required, whereas the addition of chloramphenicol ($10^{-4}M$), nitrogen mustard ($10^{-5}M$) and 2,4-dinitrophenol ($10^{-4}M$) caused no inhibitory effect upon the transfer mechanism.

2. From the time course incubations performed with minced silkglands it seems difficult to find out any particular causal relation between cell debris protein and particulate or supernate protein. And the protein-bound radioactivity in cell debris might not be associated with the transfer reaction.

3. Slices of normal and regenerating rat liver showed a relatively high incorporation of C^{14}-amino acids in to lipid fraction compared with that of the protein fraction. However, in the tissues showing excellent protein synthesis such as silkworm's posterior silkglands and rat ascites hepatoma cells, the incorporation of C^{14}-amino acids into lipids was by far at a lower rate than that of the protein. No initial marked uptake of radioactivity into lipid fraction was observed.

4. When the silkgland cell debris were preliminary treated by sodium deoxycholate, the transfer of radioactivity was markedly suppressed although their radioactivity was almost completely preserved.

Thus, as for the role of lipids in the protein synthesis in silkglands, it might be supposed that the lipid fraction is not a direct precursor of protein, but mediates the transfer reaction.

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Sulfate Reduction in *Escherichia coli*

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There are two types of sulfate reduction, the respiratory one and the assimilatory one. Sulfate reduction in sulfate-reducing bacteria belongs to the former and sulfate is utilized as the ultimate hydrogen acceptor. It was revealed by the authors (*1*) and by Peck (*2*) that adenosine-5′-phosphosulfate (APS) is an intermediate of the sulfate reduction and that cytochrome c_3 acts as an electron carrier. Higher plants and many microorganisms can utilize sulfate as the only source of sulfur and sulfate reduction in such organisms has significance in assimilating sulfur. Hilz *et al.* (*3*) and Bandurski *et al.* (*4*) studied sulfate reduction in yeast and found that another active sulfate, 3′-phosphoadenosine-5′-phosphosulfate (PAPS), is an intermediate. The formation of PAPS has been confirmed in some micro organisms, whereas PAPS reduction has not been observed in bacteria. The authors, therefore, intended to study the mechanism of sulfate reduction in *Escherichia coli* in comparison with that in sulfate reducing bacteria. The present communication describes the results obtained on the reduction of PAPS in *E. coli* with special reference to hydrogen donor systems. Mager (*5*) has also mentioned on PAPS reduction in *E. coli* by DPNH.

MATERIALS AND METHODS

Preparation of Crude Extracts—*E. Coli* B was cultured aerobically in culture medium M-9 (*6*), which contained sulfate as a sole sulfur source. Cells were washed three times with water and disrupted by sonic oscilation (10 KC) for 15 minutes. After centrifuging at 10,000×*g* for 15 minutes, the supernatant obtained was used as crude extracts, protein concentration of which was usually 30 mg. per ml.

Materials—DPNH, TPN, DPNH and lipoic acid were the products of Sigma Company. PAPS was prepared enzymatically according to Brungraber (*7*), except that precipitation procedure with Hg salt was omitted. APS was prepared chemically according to Baddily *et al.* (*8*). The amount of PAPS and APS was estimated from absorbance at 260 mμ. Dihydrolipoate ($Lip(SH)_2$) was prepared according to Hager *et al.* (*9*).

Preparation of Cytochrome c_3 and Hydrogenase—These were prepared from extracts of *Desulfovibrio desulfuricans* according to Ishimoto *et al.* (*10*).

Preparation of Glucose-6-phosphate Dehydrogenase—It was prepared from yeast according to Kornberg (*11*), except that acid precipitation was omitted.

Assay of PAPS Reduction—Reduction of PAPS was carried out anaerobically in Thunberg tubes. The reaction mixture contained 50 μmoles of phosphate buffer, pH 7.0, 0.3 μmole of PAPS, a hydrogen donor system, and an enzyme preparation in 1.5 ml. at 30°C. Reaction was stopped by the addition of 1 ml. of saturated $HgCl_2$ and sulfite in the supernatant was determined by Grant's method (*12*). In determining sulfide, reaction was carried out in Warburg vessels under the similar conditions. After incubation, hydrogen sulfide absorbed in alkali in center-wells of the vessels was determined by St. Lorant's method (*13*). In some experiments in which TPNH or methylviologen was used as a hydrogen donor, cyanide (10^{-3} *M*) was added so as to prevent further reduction of sulfite formed.

RESULTS

PAPS Reduction by Dihydrolipoate—When cell-free extracts of *E. coli* was incubated with PAPS and dihydrolipoate, accumulation of sulfite was observed (Table I). Without the extract, PAPS or dihydrolipoate, little amount of sulfite was detected. When the incubation was carried out sufficiently long,

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TABLE I

PAPS Reduction by Dihydrolipoate

	Sulfite formed
Complete system	0.143 μmole
$-$Lip(SH)$_2$	0.022
$-$PAPS	0.013
$-$PAPS+APS	0.016
$-$enzyme	0.008
$-$Lip(SH)$_2$+Cysteine	0.012
+Glutathione	0.023
+2,3-Dimercapto-1-propanol	0.036
+Mercaptoethanol	0.030

Complete system contained: 50 μmoles of phosphate, pH 7.0, 7 μmoles of Lip(SH)$_2$ or 10 μmoles each of thiols, 0.3 μmole of PAPS or 0.5 μmole of APS, 19 mg. of enzyme preparation in 1.5 ml. Incubation was carried out in hydrogen atmosphere for 2 hours at 30°C.

0.30 μmole of sulfite was formed from 0.37 μmole of PAPS. It was shown that APS could not substitute for PAPS. This indicates the absence in *E. coli* of APS reductase which is found in extracts of *D. desulfuricans.*

Other thiol compounds such as cysteine, glutathione, 2,3-dimercapto-1-propanol and mercaptoethanol, were not utilized as hydrogen donors. The fact that only dihydrolipoate is active among thiol compounds has also shown in yeast by Hilz *et al.* (*14*).

E. coli was grown in two different nutrient media: one of them contained peptone and meat extract and the other glucose, minerals but no organic sulfur. PAPS reduction was stronger in the cell grown in the latter medium than in those grown in the former. This result suggests that PAPS reduction in *E. coli* is a step to assimilate inorganic sulfate.

PAPS Reduction by TPNH or DPNH—As the crude extracts had activity to reduce DPN with ethanol and TPN with glucose-6-phosphate (G6P), ethanol and G6P with DPN and TPN, respectively, were employed as hydrogen donor systems. In both cases, PAPS reduction occurred, but the product was sulfite in the DPNH producing system and sulfide in the TPNH producing system (Table II). The difference seems to be dependent on the nature of the sulfite reducing system in the extracts. When cyanide (10^{-3} *M*), an inhibitor of sulfite reduction, was present, the reduction of PAPS was stopped at the level of sulfite even in the presence of TPNH.

TABLE II

PAPS Reduction by TPNH and DPNH

Hydrogen donor	Sulfite formed	Sulfide formed
G6P, TPN	0.055 μmole	0.12 μmole
Ethanol, DPN	0.182	0.01

Reaction mixture contained; 50 μmoles of phosphate buffer, pH 7.0, 4 μmoles of G6P or 20 μmole of ethanol, 0.1 μmole of TPN or 0.2 μmole of DPN, 0.3 μmole of PAPS, 35 mg. of enzyme. Incubation was carried out at 30°C for 1 hour.

PAPS Reduction by Methylviologen—As shown in Table III, PAPS was reduced to sulfide when the extracts supplemented with hydrogenase and methylviologen were incubated in hydrogen atmosphere.

TABLE III

PAPS Reduction by Methylviologen

	Hydrogen sulfide formed
Complete system	0.28 μmole
$-$enzyme	0.03

Complete system contained: 50 μmoles of phosphate buffer, pH 7.0, 1 μmole of methylviologen, 10 μmoles of HCOONa, 0.44 μmole of PAPS, 0.1 ml. of hydrogenase, 35 mg. of enzyme. Incubation was carried out at 30°C for 2 hours, under hydrogen atmosphere.

PAPS Reduction by Cytochrome c$_3$—In sulfate-reducing bacteria cytochrome c$_3$ participates in the reduction of APS (*1*), thiosulfate (*15*), and sulfite (*16*) as an electron carrier. It was examined whether cytochrome c$_3$ could participate in PAPS reduction in *E. coli.* As shown in Table IV, cytochrome c$_3$ was indispensable for the formation of sulfide from

Table IV

PAPS Reduction by Cytochrome c_3

	Sulfide formed	
	from PAPS	from Sulfite
Complete system	0.17 μmole	0.37 μmole
—cyt. c_3	0.05	0.02
—enzyme	0.07	0.05

Complete system contained: 50 μmoles of phosphate buffer, pH 7.0, 0.043 μmole of cyt. c_3, 0.57 μmole of PAPS or 7.5 μmoles of Na_2SO_3, 13 mg. of enzyme and 0.1 ml. of hydrogenase. Incubation was carried out at 30°C, for 120 minutes (PAPS) or 90 minutes (SO_3^{--}) under hydrogen atmosphere.

PAPS and sulfite by the crude extracts of *E. coli* supplemented with hydrogenase in hydrogen atmosphere. This does not necessarily mean that cytochrome c_3 which might be present naturally in *E. coli* in small amounts, though not yet discovered, played a role of the intermediary electron carrier in the reduction of PAPS in this case. Passage of the extracts through a column of a cation-exchange resin, Amberlite IRC-50 NH^+_4 type, which could remove cytochrome c_3 from solutions, did not depress the reduction of PAPS with TPNH producing system or dihydrolipoate (Table V). The possibility, however, is not eliminated that hemeproteins other than cytochrome c_3 with low potentials participate in the reduction as electron carriers.

Table V

Effect of IRC-50 Treatment on PAPS Reduction

Hydrogen donor	IRC-50 treatment	Sulfite formed	Sulfide formed
Lip(SH)$_2$	—	0.228 μmole	
	+	0.222	
G6P, TPN	—		0.17 μmole
	+		0.19

Composition of reaction mixtures was the same as described in Tables I, II, except enzyme added was 6 mg. and 30 mg.

Effects of Quinacrine on PAPS Reduction—Quinacrine inhibited PAPS reduction by 70 per cent at $10^{-3}\,M$ when methylviologen or TPN was used as an electron carrier, whereas hydrogenase or G6P dehydrogenase were not inhibited at this concentration. On the contrary, the reagent had little effect on PAPS reduction by dihydrolipoate (Table VI). It follows from this result that an additional enzyme or enzyme systems which can be inhibited by quinacrine are required for the PAPS reduction by TPNH or methylviologen when compared with that by dihydrolipoate.

Table VI

Effect of $10^{-3}\,M$ Quinacrine on PAPS Reduction

Hydrogen donor	Inhibition
Lip(SH)$_2$	10%
G6P, TPN	74
Methylviologen	85

Composition of the reaction mixtures was the same as that described in Tables I, II, III, respectively except for addition of $10^{-3}\,M$ KCN and of $10^{-3}\,M$ quinacrine.

Purification of PAPS Reductase—Purification of dihydrolipoate-linked PAPS reductase was intended so as to elucidate the nature of the hydrogen donor system. Crude extracts (77 ml.) from 18 g. of wet cells was brought to 0.33 saturation by the addition of solid ammonium sulfate. After centrifugation, the supernatant brought to 0.67 saturation and precipitate was collected and dissolved in 0.05 M phosphate buffer, pH 7.0. The enzyme solution was dialysed against distilled water overnight. This preparation was called P33-67. Three gramme of calcium phosphate gel was added to the dialysed solution to adsorb the enzyme. Elution was carried out with 50 ml. of 0.125 M phosphate buffer, pH 7.0, twice (GE). Specific activity of GE was about 6 times higher compared with that of crude extracts, as indicated in Table VII. After dialysis, GE was applied to DEAE-cellulose column and elution was carried out by gradient change of solvent concentrations. The mixing chamber was filled with 0.01 M

TABLE VII

Results of Purification Procedures

Purification step	Specific activity (unit/mg.protein) when following hydrogen donors were used			
	Lip(SH)$_2$	Methyl-viologen	G6P, TPN	DPNH
Crude extract	13	11	9.4	
P33-67	27	22		
GE	75	39		
DEAE-cellulose	79	0	0[1)]	0[2)]

Purification procedure was described in the Text.

[1)] G6P dehydrogenase preparation which reduced 0.1 μmole of TPN per minutes was added.

[2)] 1 μmole of DPNH was added.

phosphate buffer, pH 7.0, and the upper chamber was filled with 0.25 *M* phosphate buffer, pH 7.0. Protein was eluted in two peaks and the second broad one was lyophilysed. The specific activity of this preparation when measured with dihydrolipoate was not much raised, but it is noteworthy to mention that none of methylviologen, G6P and TPN nor DPNH was active as a hydrogen donor in this preparation.

Effect of Inhibitors—Effects of various inhibitors on PAPS reduction by dihydrolipoate were shown in Table VIII. Enzyme was preincubated with inhibitors for 10 minutes before PAPS and dihydrolipoate was added. It is not strange that heavy metals and SH reagents had little effect. Heavy metals and SH reagents may be removed by dihydrolipoate. Monoiodoacetic acid which combines irreversibly with the enzyme showed strong inhibition, but it is not clear whether the enzyme has essential SH or amino groups.

TABLE VIII

Effect of Inhibitors on PAPS Reduction

Added substance	Concentration	Inhibition
KCN	1 m*M*	0
NaN_3	1	0
α,α′-Dipyridyl	1	0
Quinacrine	1	10
CH_2ICOOH	1	73
PCMB	0.5	14
$NaAsO_2$	1	6
$AgNO_3$	0.1	0
$CuSO_4$	0.1	0
$CaCl_2$	10	20
APS	0.5	0
ATP,ADP,AMP,	2.5	0

Enzyme was preincubated with inhibitors for 10 minutes before PAPS and Lip(SH)$_2$ were added.

DISCUSSION

It is accepted that PAPS is an intermediate of assimilatory sulfate reduction, whereas the hydrogen donor system of PAPS reduction remained to be elucidated. Bandurski *et al.* (*17*) reported that in yeast a peptide containing SH groups acted as an intermediary electron carrier between TPNH and the reductase. Hilz *et al.* (*18*), on the other hand, presented a scheme including lipoic acid and reported that dihydrolipoate was used as a hydrogen donor (*14*). In *E. coli*, Mager (*5*) observed that PAPS was reduced by DPNH rather than TPNH but fine mechanism was not presented. The present authors also found reduction of PAPS by DPNH, TPNH and dihydrolipoate in *E. coli* extract, in accordance with the results obtained by Hilz in yeast.

The results of the inhibition by quinacrine, known as a specific inhibitor of flavoproteins, suggest participation of additional enzymes in PAPS reduction by TPNH and methylviologen when compared with the enzyme system having dihydrolipoate as a hydrogen donor. Recently, a flavo-enzyme, lipoate dehydrogenase was found to occur in various organisms, including *E. coli* (*19*). The extracts of *E. coli* obtained by the authors had activity to reduce lipoate by TPNH. However, the possibility that one of the additional enzymes might be lipoate dehydrogenase and that free lipoate might play a role of the intermediary electron carrier were excluded by the fact that the addition of lipoate did not stimulate PAPS reduction by TPNH or methylviologen even in the dialysed extracts. Participation of lipoic acid bound to proteins or SH containing peptides or proteins as

Bandurski reported may be considered.

In view of these results, the following scheme is presented.

TPNH
DPNH ——Flavoprotein——PAPS reductase——PAPS
Methylviologen |
Dihydrolipoate——|

The enzymic mechanism of sulfate reduction in *E. coli* described here was fundamentally similar to that of sulfate reduction in yeast, not only in regard to the active sulfate intermediate but also in regard to the electron carrier system. Comprehensive comparative studies are expected in various organisms of the assimilatory and the respiratory types.

SUMMARY

1. Extracts of *E. coli* was found to reduce adenosine-3′-phospho-5′-phosphosulfate by TPNH, DPNH, methylviologen and dihydrolipoate.

2. Cytochrome c_3 of *Desulfovibrio desulfuricans* could be also utilized as a hydrogen donor.

3. From experiments of inhibition of quinacrine and of purification, dihydrolipoate was found to be a direct hydrogen donor in PAPS reduction.

The authors should like to express their thanks to Prof. F. Egami for his valuable advices.

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Studies on Xylanase of *Pericularia oryzae*

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Pericularia Oryzae is known to cause a disease of the rice plant. Since the mycelia of this fungus penetrate the cell membrane of the host easily, excretion of enzymes which decompose the constituents of the membrane should be expected. Presence of xylanase, proteinase, amylase and cellulase has been demonstrated (*1*).

It was found that *Pericularia oryzae* strain No 9 excretes two kinds of xylanase. It is the purpose of the present paper to describe the preparation and properties of these two distinct enzymes.

EXPERIMENTALS

Materials and Analytical Procedure—Xylan was prepared by Hibino's method (*2*) extracting rice straw with 7% NaOH and subsequent precipitation of the extract with 50% ethanol. The preparation was free from cellobiose and starch as confirmed by the paper chromatography of the *N* HCl hydrolysate. DEAE-cellulose used for the enzyme purification was prepared by Peterson and Sober's method (*3*). Celite No. 535 used in the active carbon chromatography was the product of the Johns Manville Company and the active carbon was Shirasagi A of the Takeda Chemicals Company. Xylose was a commercial product of Wako Pure Chemical Corporation. The enzyme activity was assayed by estimating increase of reducing sugar. Two ml. of 0.1 *M* phosphate buffer, pH 8.1 or pH 6.4, 0.1 ml. of 0.003 *M* calcium acetate, 1 ml. of 2% xylan and enzyme solution were incubated at 45°C for 15 minutes. The reaction was stopped by immersing aliquots of the mixture in boiling water for 5 minutes. The mixture was deproteinized by Somogyi's method (*4*) and the reducing sugar formed was assayed by Nelson's arsenomolybdate method (*5*). The values obtained with a control tube consisting of the above mixture except the addition of xylan was subtracted from the experimental values. A unit of enzyme is defined as the amount which causes a formation of 1.0 mg. of reducing sugar for 15 minutes. Specific activity is expressed as units per milligram of protein. Protein was assayed by Lowry's method (*6*). Paper chromatography was carried out by the ascending technique with aqueous phenol as a solvent system. Aniline phthalate and orcinol spray were used to detect glucose and xylose, respectively.

Growth of P. oryzae and Preparation of Xylanase—The fungus was cultivated at 28°C for a week in the following medium; sucrose 1.5 g., xylan 1.5 g., KH_2PO_4 1.0 g., K_2HPO_4 3.0 g., KNO_3 3.0 g., urea 0.5 g., $CaCl_2 \cdot 6H_2O$ 0.1 g., $MgSO_4 \cdot 7H_2O$ 0.5 g., $FeSO_4$ 0.75 mg., $MnSO_4 \cdot 5H_2O$ 0.22 mg., $ZnCl_2$ 7.5 mg., H_3BO_3 0.07 mg., $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.06 mg., $CuSO_4 \cdot 5H_2O$ 0.06 mg., biotin 1 μg., thiamine 1 mg. per liter (*7*).

After filtering off the mycelia, the filtrate was added with calcium acetate (1 *M*, pH 6.0) to a final concentration of 0.02 *M* and pH was adjusted to 6.0. The precipitate formed was removed by centrifugation for 10 minutes at $3{,}000 \times g$. Five g. of alumina $C\gamma$ was added to 50 ml. of the supernatant (0.328 mg. protein/ml.). After $C\gamma$ which absorbed enzyme protein had been washed with 5.0 ml. of 0.1 *M* phosphate buffer, pH 7.0, the enzyme was eluted from

Table I

Purification of Xylanase

Fraction	Activity units/ml.	Specific Activity units/mg.	Yield %
Medium of Culture	0.42	0.8	100
Treatment of Ca-acetate (supernatant)	0.39	2.7	94
$C\gamma$ gel eluent (phosphate buffer pH 9.0, 0.1 *M*)	18.1	16.4	86
$(NH_4)_2SO_4$ fraction (0.4–0.5 saturation)	14.5	20.2	67

Cγ with 20 ml. of 0.1 *M* phosphate buffer, pH 9.0. This eluate was fractioned with ammonium sulfate between 40 and 50% saturation. The precipitate thus obtained was dissolved in 20 ml. distilled water. This preparation was used as the enzyme solution in the following experiments. Table I summarizes a typical purification procedure, in which 25 fold purification was attained.

RESULTS AND DISCUSSION

Effect of pH and Temperature—The enzyme preparation had two values of pH optimum for xylanase activity, one at pH 8.1 and the other at pH 6.4 (Fig. 1).

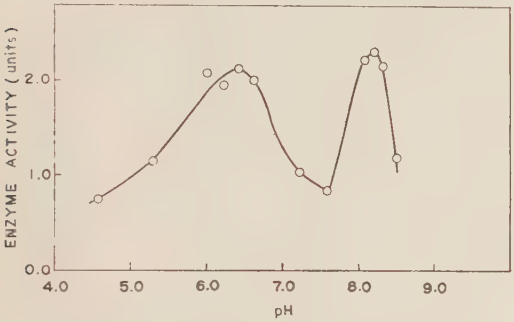

Fig. 1. Effect of pH on enzyme activity.

Each reaction mixture contained 0.1 ml. of enzyme (Sp. Act. 25) 0.1 ml. of Ca acetate, 1 ml. of 2% xylan and 2 ml. of 0.1 *M* phosphate buffer of respective pH. The incubation was carried out at 45°C for 15 minutes. The enzyme activity was assayed as described in the text.

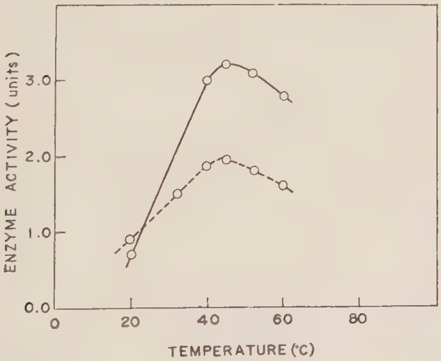

Fig. 2. Effect of temperature on enzyme activity.

The enzyme activity was assayed as described in the text. The reaction mixtures were preincubated at the respective temperature for 5 minutes before the addition of 0.1 ml. of the enzyme preparation. Solid line: pH 6.4 xylanase, broken line: pH 8.1 xylanase

The optimum temperature for both pH 6.4 xylanase and pH 8.1 xylanase activities is found to be 45°C, as is shown in Fig. 2.

Effect of Substrate and Enzyme Concentration—The reaction rate increased with increasing amount of xylan (0 to 1 mg.) as was shown in Fig. 3.

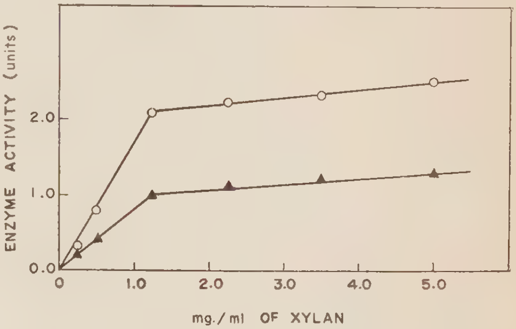

Fig. 3. The influence of xylan concentration on the enzyme activity.

The reaction conditions were similar to those in Fig. 1 experiment except the concentration of xylan. The concentration of enzyme used was 1.5 mg./ml. (Sp. Act. is 25). —○— pH 6.4 xylanase, —▲— pH 8.1 xylanase.

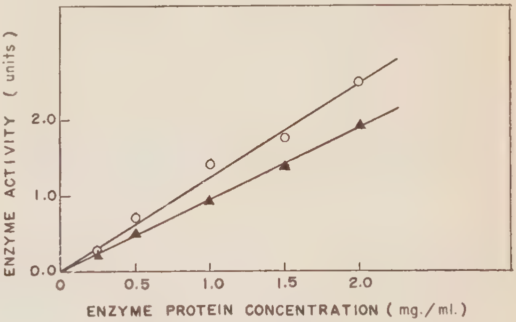

Fig. 4. Effect of the enzyme concentration on the enzyme activity.

The reaction conditions were similar to those in Fig. 1 experiment except the concentration of the enzyme. Specific activity of the enzyme used was 25 units/mg. protein. —○— pH 6.4 xylanase, —▲— pH 8.1 xylanase.

Fig. 4 indicates the results obtained with varying enzyme concentration in the presence of 2 mg./ml. of xylan. The rate of reaction was proportional to the enzyme concentration in the whole range tested.

The Stability of the Enzyme Activity at Various pH and Temperatures—As shown in Fig. 5, pH 8.1 xylanase is stable in the pH range between 5.78 and 10.0, while the pH 6.4 xylanase is stable in the pH range between 3.0 and 8.0.

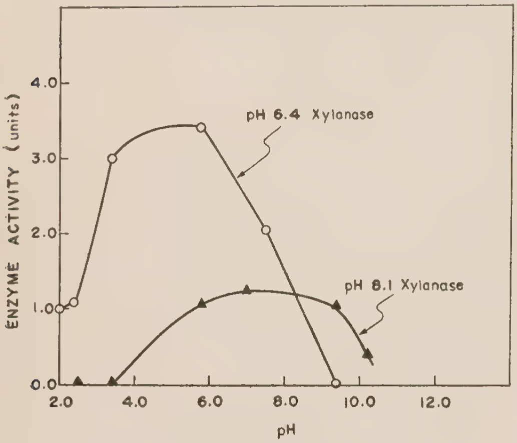

FIG. 5. The stability of the enzyme activity at various pH.

The incubation was carried out for 2 hours with phosphate buffer, 0.1 *M*, between pH 5.5 and 10.0; citrate buffer, 0.1 *M*, between 5.5 and 2.2. After pH was readjusted to pH 6.4 and pH 8.1 respectively with 0.1 *M* NaOH or 0.1 *M* H_3PO_4, enzyme reaction was carried out as described in the text.

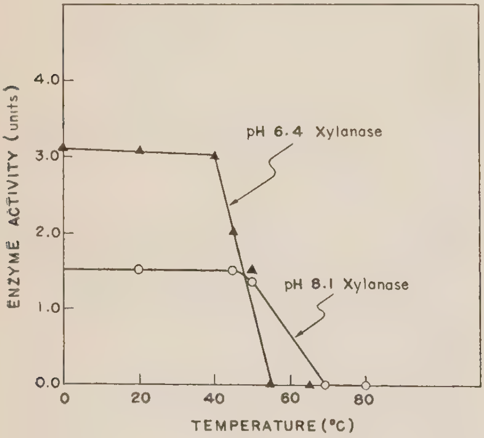

FIG. 6. The stability of both enzymes at various temperatures.

Each enzyme was incubated in 0.1 *M* phosphate buffer, pH 6.4 and 8.1, for 2 hours. After incubation, the substrate was added and assayed as described in the text.

As shown in Fig. 6, pH 8.1 xylanase was stable at the temperature range between 0° and 50°C, and pH 6.4 xylanase was stable between 0° and 40°C.

The Effect of Metal Ions for Xylanase Activity—The effect of metal ions for the enzyme activity was studied. The results obtained are presented in Table II.

TABLE II

Effect of Metals on Xylanase Activity

Metal ions added	Concentration *M*	Activity	
		pH 6.4 Xylanase units/ml.	pH 8.1 Xylanase units/ml.
None	—	2.0	1.0
Ca^{++}	10^{-5}	12.0	7.0
Ca^{++}	10^{-4}	17.0	11.0
Ca^{++}	10^{-3}	19.0	13.0
Mg^{++}	10^{-3}	9.0	4.0
Zn^{++}	10^{-3}	0.0	0.0
Fe^{++}	10^{-3}	0.0	0.0
Co^{++}	10^{-3}	0.0	0.0
p-CMB	10^{-5}	0.0	0.0

The incubation mixture consisted of 0.3 ml. of enzyme (Sp. Act. of 30); 0.1 *M* phosphate buffer of pH 8.1 or pH 6.4 and metal ions. Ca was added as acetate; Fe as Mohr's salt; and other as chloride.

Both enzymes were found to be activated by Ca^{++} ions (10^{-3} *M*), while Zn^{++}, Fe^{++} and Co^{++} ions of the similar concentration range were found to inhibit these enzyme activities. *p*-CMB (10^{-3} *M*) completely inhibited both enzyme activities.

Reaction Product by Xylanase—The reaction product was determined by the procedures that follow below. Two ml. of 2% xylan was incubated with 4 ml. of 0.1 *M* phosphate buffer, pH 8.1 or pH 6.4, 0.3 ml. of 0.003 *M* calcium acetate and 3 ml. of enzyme at 45°C for 30 minutes. The reaction was stopped by immersing the reaction mixture in boiling water for 5 minutes. The precipitate formed was removed by centrifugation at 16,000×*g* for 15 minutes. The supernatant fluid was treated with Amberlite IR 120, H form, and Dowex-1, OH-form, to remove salts. Nine ml. of salt free supernatant was concentrated to 3.0 ml. under 22 mm. Hg pressure at 70°C. The concentrated fluid was chromatographed by the method of Wadman (*8*). The chromatograms obtained were presented in Fig. 7.

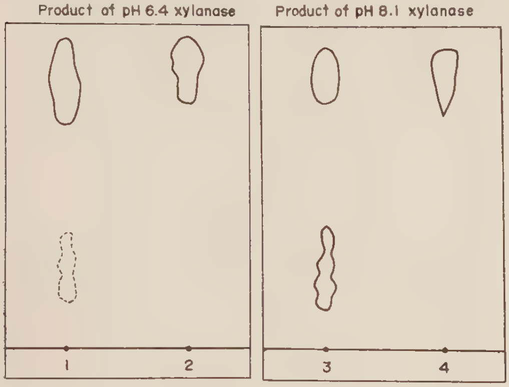

Fig. 7. Chromatography of the product of xylanase action.

1 is the enzymatic reaction product of pH 6.4 xylanase. 3 is that of pH 8.1 xylanase. 2 and 4 are the standard xylose. The conditions of enzymatic reaction and the procedure of concentrating enzymatic product was described in the text. The solvent front was 16 cm. from the origin.

In the paper chromatogram of the product by pH 6.4 xylanase (left, No. 1), a clear fluorescent spot was observed at the same position of authentic xylose. However, three other obscure spots were also found in this chromatogram. The chromatogram of the product formed by pH 8.1 xylanase is also shown in Fig. 7 (right, No. 3). The spot with the highest R_f value was identified as xylose because it corresponded to the authentic xylose (No. 4), but the other spots were not identified. When pH 6.4 xylanase prepared with DEAE-cellulose was used, the only spot which was detected corresponded to that of authentic xylose.

Chromatography of the products by active carbon was carried out by Whistler's method (*9*). Ten ml. of 2% xylan were incubated with 4 ml. of 0.1 *M* phosphate buffer, either pH 8.1 or pH 6.4, 0.6 ml. of 0.003 *M* Ca-acetate and 3 ml. of the enzyme solution at 45°C for 1.5 hours. The reaction mixture was treated and concentrated by the same procedure as described above and the concentrate was chromatographed on the active carbon column. The results were presented in Fig. 8.

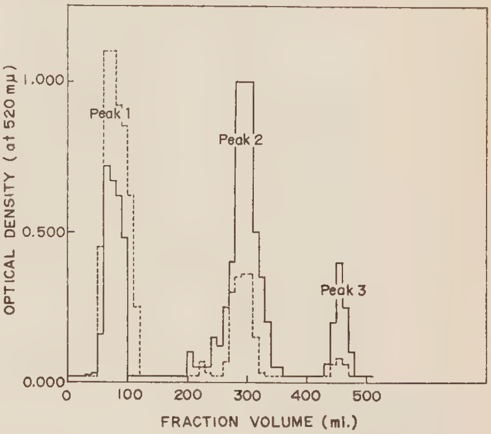

Fig. 8. Active carbon chromatogram of the products of pH 6.4 xylanase and pH 8.1 xylanase.

The concentrate as described in the text was run through a 1×15 cm. column of Takeda Shirasagi A (active carbon) and Celite 535 mixture (1 : 1 weight). The adsorbed compounds were subjected to the gradient elution of 15% ethanol-H_2O at a rate of 10 ml. per hour. Optical density (O. D.) was measured at 520 mμ of Nelson's sugar assay method. Solid line is pH 8.1 xylanase, broken line is pH 6.4 xylanase.

The substance of the peak 1 was identfied as xylose by paper chromatography. When the peak 2 eluate was hydrolysed by *N* HCl for 2 hours, the hydrolysate showed twice as much reducing power as the original eluate and contained only xylose as shown by the paper chromatogram. These facts suggest the presence of xylobiose in peak 2. When the material representing peak 3 had been hydrolyzed with *N* HCl for 2 hours, the reducing power of this solution was observed to increase 3.2 times more than that of the initial material and in this hydrolysate xylose was the only compound detected by paper chromatography. The presence of xylotriose was suggested from those results.

Chromatography of Xylanase with DEAE-Cellulose—Those experiments were carried out to ascertain whether two different forms of xylanase were excreted by *Piricularia oryzae* Strain No. 9 or not. The enzyme preparation, fractionated with ammonium sulfate (40–50% saturation), was dialyzed against distilled water containing 0.001 *M* Ca-acetate for 20 hours. An aqueous slurry of 3.0 g. of DEAE-cellulose was packed into a glass column, 1×20 cm. Ten ml. of enzyme solution were poured on to the column. A gradient elution was carried out with 0.1 *M* phosphate buffer, pH 7.0, in a mixing flask, and 0.1 *M* phosphate, pH 7.0, containing 0.1 *M* NaCl in reservoir. The column flow was at the rate of approximately 8 ml. per hour. Fig. 9 shows the correlation between the protein content of each fraction and either pH 8.1 xylanase or pH 6.4 xylanase activity.

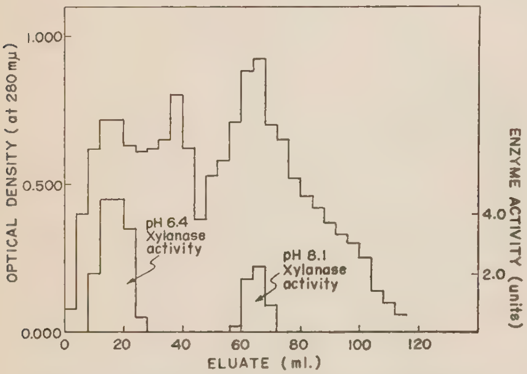

Fig. 9. Chromatography of xylanase (pH 6.4 and pH 8.1) fractionation with DEAE-cellulose column.

The optical density was read at 280 mμ. Assay for xylanase activity was carried out with 2 ml. of 0.1 *M* phosphate buffer, pH 6.4 or pH 8.1, 0.1 ml. of 0.003 *M* Ca-acetate, 1.0 ml. of xylan, 0.1 ml. of enzyme at 45°C for 15 minutes. Assay procedure and unit of enzyme activity were defined in the text.

The specific activity of pH 6.4 xylanase increased from 20.2 to 66, while the specific activity of pH 8.1 xylanase remaind at 18. pH 8.1 xylanase was found to be less stable than pH 6.4 xylanase. The modifications of the chromatography, such as the change of the composition of eluting solvent, the addition of calcium ion, the application of an anaerobic condition with N_2 gas or cooling of the column in an ice bath, specific activity of pH 8.1 xylanase could not be raised and the enzyme was sometimes completely inactivated during the process. DEAE-cellulose chromatography was considered not suitable for the preparation of pH 8.1 xylanase.

The Relation between Growth of P. oryzae and Formation of Xylanase in the Medium with Various Carbon Source—Table III shows activity of

Table III

The Formation of Xylanase, Amylase and Cellulase by P. oryzae in the Media Containing Different Carbon Sources

Carbon source	Quantity of C-compd (g./liter)	Weight of fungus grown (g./liter)	Activity (unit/ml.)		
			Xylanase	Amylase	Cellulase
Sucrose	15	54	0.2	8	0.1
Rice straw	330	58	0.5	8	0.1
Xylan plus sucrose	10 plus 5	56	15.0	8	0.1
Soluble starch	15	80	0.2	28	0.1
Carbozymethyl Cellulose	15	3	0.1	0.2	0.1
Xylan	15	56	24.0	10	0.1

The assay system consisted of 2 ml. of 0.1 *M* phosphate buffer, pH 7.0, 0.1 ml. of 0.003 *M* Ca-acetate, 1 ml. of culture medium, and 1.0 ml. of 2% solution of carbohydrates indicated; xylanase; xylan, amylase; soluble starch, cellulase; carboxymethylcellulose.

polyases of *P. oryzae* Strain No. 9 culture media of different carbon sources. The activity of xylanase and amylase varies remarkably with the difference of carbon source, while cellulase activity remains always constant.

The reaction mixture was incubated for 30 minutes at 45°C. The increased reducing sugars were measured with Nelson's method (*5*). Unit was defined in the text.

Since the enzyme was prepared from the organisms grown in the medium containing xylan as a sole carbon source, xylanase is considered to be an adaptive enzyme in this strain.

SUMMAY

Two different xylanases were identified in the culture medium of *Piricularia oryzae* Strain No. 9. The optimum pH values of these enzymes were 8.1 and 6.4, respectively.

The optimum temperature for pH 6.4 enzyme is 45°C. It is stable between pH of 6.0 to 3.5 at 45°C and between temperatures from 0° to 40°C at pH 6.4. The Ca^{++} ions, 10^{-3} *M*, activates this enzyme markedly, while 10^{-3} *M* Zn^{++}, Co^{++}, Fe^{++} ions and 10^{-5} *M* *p*-CMB are inhibitory. The product of the reaction is identified chromatographically as xylose.

The properties of pH 8.1 xylanase is as follows; The optimum pH is 8.1, the optimum temperature is 45°C. This enzyme is stable between pH 6.0 and pH 9.5 at 45°C, and from 0° to 50°C at the optimal pH. The activation by Ca^{++} ion at a concentration of 10^{-3} *M*, and the inhibition by 10^{-3} *M* Zn^{++}, Co^{++} and Fe^{++} ions and 10^{-5} *M* *p*-CMB are also observed like pH 6.4 xylanase. It seems that the latter enzyme degrades xylan to the fragments of oligosaccharides such as xylobiose and xylotriose.

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NOTES

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Ribonuclease I Digestion of Yeast S-RNA*

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(Received for publication, July 3, 1961)

The importance of S-RNA in protein synthesis is now generally accepted. The present paper deals with the digestion of yeast S-RNA by RNase I. (pancreatic RNase).

TABLE I

Base Composition of Yeast S-RNA and Base Analysis of Several RNase I Digestion Products

Nucleotide	mole %	A=10.0		Distribution	
			Sequences	% of total cytidylic acid	
				determined	calculated[1]
C	26.6	13.9	PyC	49.5	51.5
			PyAC	6.5	9.9
			PyAAC	1.8	1.9
			PyGC	9.8	15.1
A	19.2	10.0		% of total adenylic acid	
				determined	calculated
			PyAC	9.9	13.7
			PyAAC	5.5	5.3
G	29.3	15.3		% of total guanylic acid	
				determined	calculated
			PyGC	10.0	13.7
U	20.1	10.5		% of total uridylic acid	
				determined	calculated
			PyU	43.7	51.5
5RU	4.8	2.5		% of total 5RU	
				determined	calculated
			Py5RU	44.8	51.5

1) On the basis of random distribution

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* Abbreviations: RNA, ribonucleic acid; RNase, ribonuclease; S-RNA, soluble RNA; Tris, tris (hydroxymethyl) aminomethane; C, cytidylic acid; A, adenylic acid; G, guanylic acid; U, uridylic acid; 5-RU, 5-ribosyl uracil nucleotide; Py, pyrimidine nucleotides; AC, adenylyl cytidylic acid; AAC, adenylyl cytidylic acid; GC, guanylyl cytidylic acid.

Soluble RNAs were prepared from pressed fresh baker's yeast (purchased from a local store) without grinding.

Monie's modification (*1*) of Kirby's method was applied with further slight modification: phenol treatment followed by alcohol precipitation, extraction with *N* NaCl and adsorption on ECTEOLA-cellulose.

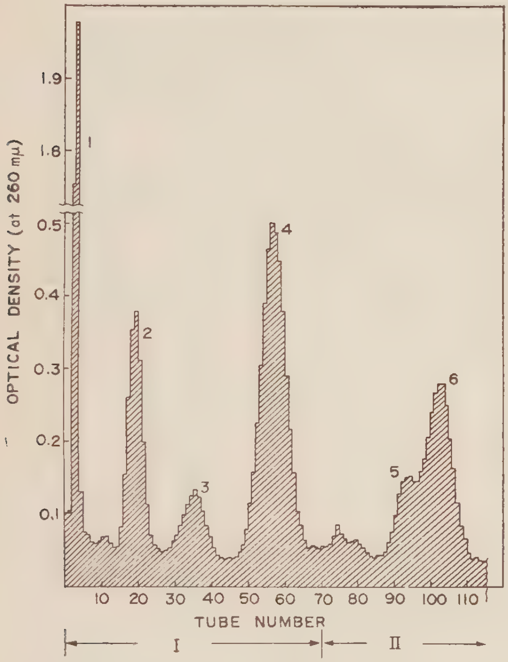

Fig. 1. Chromatographic separation of the products of RNase I digestion of S-RNA from yeast.

Exchanger: Dowex-1 X-2 chloride, 200- 400 mesh, 0.8×11 cm. Solution: I, 0.007 *N* HCl; II, 0.01 *N* HCl. One tube contains 8.1 ml.

S-RNA thus obtained has an ability to accept 5.1 μg. (0.0325 μmoles as leucine) of amino acids mixture (hydrolysate of Chlorella protein) per mg. of RNA.* It seemed, consequently, that almost all molecules of the S-RNA could accept one molecule of amino acid.

Digestion by RNase I was carried out as follows: 13.0 mg. of the S-RNA, 0.2 mg. of RNase I, 0.2 ml. of 1.0 *M* Tris buffer (pH 7.6) and water to final 2.0 ml. are incubated at 37°C for 6 hours. Then the cyclic nucleotides formed as intermediates were splitted by the overnight treatment in 0.1 *N* HCl at 2°C. After neutralization (to pH 8) the digestion mixture was diluted to 30 ml. and subjected to a column of Dowex-1 X-2 chloride form. The elution was carried out according to the method of Volkin and Cohn (*3*). Nucleotide composition of the soluble RNA was determined by Dowex-1 X-2 formate type, after alkaline hydrolysis as described by Osawa *et al.* (*4*).

The results are shown in Figure 1 and Table I. In the figure, peaks 1, 2, 3, 4, 5 and 6 are identified as C, AC, 5RU, U, AAC and GC, respectively, by their ultraviolet absorption spectra in acid and alkaline regions. In the Table partial distribution of nucleotides is shown. It seems that 5RU is distributed in the same manner as that of U and that nucleotide distribution so far observed by the analysis of several digestion products is shown to be not so far from that calculated as random distribution.

The author wishes to express her gratitude to Prof. F. Egami for his helpful advice.

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* Assay was according to the method of Osawa (*1*).

The Journal of Biochemistry, Vol. 50, No. 6, 1961

Polysulfated Mucopolysaccharides of Elasmobranch Cartilage

The occurrence in shark cartilage of a chondroitin sulfate containing more than one sulfate per acetylgalactosamine residue had been reported by Soda *et al.* (*1*), Utimaru (*2*) and Suzuki (*3*). Although the isolation of a disulfated disaccharide from the preparation (*3*) has clearly indicated the polysulfation, many questions remain unanswered: for example, a possibility that the disulfated disaccharide was derived from impurities which contained a very high proportion of disulfated disaccharide has not yet been excluded: nor has the distribution of the compound in *Elasmobranchii* been illustrated.

A number of sulfated mucopolysaccharides of cartilage have been prepared from various species of shark and characterized in this laboratory (*4*, *5*). The most interesting is the finding that there are a chondroitin sulfate and a kerato sulfate found in the cartilage of *Scoliodon walbechmi*, both of which contain more than one sulfate residue per repeating unit. Such polysulfated compounds have not been isolated from the other species so far investigated. Analytical data of the preparations are shown in Table I. The same substances are obtained from further fractional precipitation with ethanol of each fractions respectively.

The polysulfated chondroitin* was precipitated as Ca-salt with ethanol (*4*) between 38–40% concentrations, $[\alpha]_D=-17.0^\circ$ (2 per cent in water, Na-salt). Infrared spectra showed bands at 828, 1240–1255 and 1560 cm^{-1}, all of which are similar to those previously found in chondroitin sulfate C (*11*, *12*). Hydrolysis of the compound yielded galactosamine (20% HCl at 100°C for 15 hours) and glucuronic acid (N H_2SO_4 at 100°C for 14 hours). The galactosamine-HCl was crystallized by the method of Roseman and Ludowieg (*13*) and shown to be of D-configuration ($[\alpha]_D=+94^\circ$, 1 per cent in water, final). The glucuronic acid was identified by the method of Khym and Doherty (*14*).

Table I

Analytical Data of Polysulfated Mucopolysaccharides

	Chondroitin Polysulfate (Na-salt) %	Kerato Polysulfate (Na-salt) %
Ester-sulfate (SO_4)[1]	21.5	28.7
Nitrogen[2]	2.45	2.74
Hexosamine[3]	31.5	31.2
Uronic Acid[4]	33.1	less than 2.4
Galactose[5]	less than 2.0	31.4
N-Acetyl[6]	8.8	6.6
Ash	30.8	22.2

1) Estimated by the method of Anderegg *et al.* (*6*).

2) Estimated by the micro Kjeldahl method.

3) Estimated by the modified Elson-Morgan method (*7*).

4) Estimated by the carbazole reaction (*8*).

5) Estimated by the anthrone reaction (*9*).

6) Estimated by the method of Ludowieg and Dorfman (*10*).

The polysulfated analogue of kerato sulfate was obtained by the ethanol fractionation (45–65% concentrations). $[\alpha]_D=+5.4^\circ$ (2 per cent in water, Na-salt). Infrared spectra showed bands at 825, 1230–1265 and 1560 cm^{-1}. The band at 1615–1635 cm^{-1}, which could be assigned to uronic acid, was not seen in this spectrum. From the acid hydrolysate of this compound, a crystalline hexosamine-HCl was obtained (*13*), and identified as D-glucosamine-HCl, ($[\alpha]_D=+72°$ 1.6 per cent in water, final). Approximately equivalent amounts of D-galactose were also obtained from the hydrolysate and identifid by paper-chromatography. α-Methylphenlhydrazone of the galactose (*15*) has also been prepared; m. p. 177–178°C (uncorrected), mixed m. p. 177–179°C.

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* Recently, the occurrence of a polysulfated chondritin has also been confirmed by Dr. K. Meyer, Columbia University (Personal communication to Dr. F. Egami).

The author wishes to express the sincere thanks to Drs. F. Egami, S. Suzuki and T. Soda for their valuable discussions and encouragements during these studies.

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LETTERS TO THE EDITORS

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Synthesis of Adenylyl 2,3-Diphosphoglyceric Acid

An adenine nucleotide identified as adenylyl 2,3-diphosphoglyceric acid has been found in blood of some animals (*1–3*). It is the purpose of this communication to describe the synthesis of this compound and to compare the product with the naturally occurring nucleotide.

4-Morpholine N, N'-dicyclohexylcarboxamidium adenosine-5'-phosphoromorpholidate (0.1 mmole) prepared by the method of Moffat and Khorana (*4*) and bis-(tri-*n*-butylammonium) 2,3-diphosphoglyceric acid (1 mmole) were dissolved in an anhydrous pyridine (5 ml.). The reaction mixture was shaken mechanically at room temperature. After 6 or 8 hours, the mixture was diluted with water, brought to pH 8 with ammonium hydroxide and applied to a column of Dowex 1 (formate) resin. After washing with water the column was eluted using a modified formic acid system (*2*). The effluent pattern of the synthetic adenylyl diphosphoglyceric acid was quite equal to that of the pig blood nucleotide, when the P^{32}-labelled synthetic compound and the non-labelled pig nucleotide were cochromatographed. The use of tri-*n*-butylammonium salt resulted in a higher yield than that of free diphosphoglyceric acid (average yield : 0.03 mmole, based upon the morpholidate). The synthetic product was isolated as the barium salt from the eluates.*

Anal. Found; Adenine : ribose : labile P : total P : glyceric acid = 1.00 : 0.97 : 0.14 : 2.90 : 0.99 Theor.; 1 : 1 : 0 : 3 : 1

When chromatographed on paper using various developing solvents, both of the nucleotide (natural and synthetic) had equal R_f values.

The acid prostatic phosphatase was used to clarify the molecular configuration of the nucleotide. The enzyme was purified by the method of Boman (*5*) and freed from inorganic phosphate by the Gel-filtration method (Sephadex G-25, previously washed with 0.01 % Tween 80 solution). But the phosphatase did not liberate inorganic phosphate from both the natural and the synthetic nucleotide**.

TABLE I

Rf Values of the Nucleotides

Compound	*Rf* values in solvent I	II	III
Adenosine-5'phosphate	0.57	0.25	0.30
Adenosine-5'diphosphate	0.50	0.18	0.35
Adenosine-5'triphosphate	0.40	0.14	0.38
Adenosine-5'phosphoromorpholidate	0.86	—	—
Adenylyl monophosphoglyceric acid	0.48	—	—
Adenylyl diphosphoglyceric acid			
Synthetic	0.33	0.10	0.42
Isolated from			
Pig blood	0.32	0.10	0.42
Human blood	0.32	0.10	0.42
Rabbit blood	0.32	0.10	0.42

Ascending paper chromatography on Whatman No. 1 paper was carried out in the following solvents (*2*): Solvent I, *n*-butyric acid-0.68 *N* sodium hydroxide (69 : 31); Solvent II, *n*-propyl alcohol-conc. ammonium hydroxide-water (20 : 10 : 3); Solvent III, 0.1 *M* phosphate buffer at pH 6.8-ammonium sulfate-*n*-propyl alcohol (100 : 60 : 2). The compounds were located by observation under an ultraviolet lamp.

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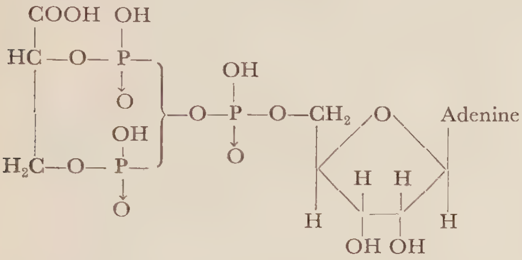
[*] According to a personal communication, A. M. Michelson had synthesized the lithium salt of the nucleotide with the use of P^1-adenosine-5'P^2-diphenyl pyrophosphate (Yield; 60–70 per cent of the pyrophosphate anhydride.)

[**] Phosphoglycolic acid, mono- and di-phosphoglyceric acid, 5-phosphoribonic acid and 6-phosphogluconic acid were hydrolyzed very slowly compared to *p*-nitrophenyl phosphate. Relative rate of hydrolysis of these compounds at pH 5.0 or 5.5, were only a few per cent of *p*-nitrophenyl phosphate. ATP and bis-(*p*-nitrophenyl) phosphate were not hydrolyzed (less than 0.01 per cent). It is interesting that phosphoglycolic acid, mono- and di-phosphoglyceric acid did not inhibit the hydrolysis of *p*-nitrophenyl phosphate but 6-phosphogluconic acid showed an inhibitory effect to the enzyme action (see (*6*)).

The use of the intestinal alkaline phosphatase was also unsuccessful, that is, the nucleotide was hydrolyzed first by the contaminating phosphodiesterase activity and the products were immediately dephosphorylated by the phosphomonoesterase.



Adenylyl monophosphoglyceric acid was synthesized with use of tri-*n*-butylammonium-3-phosphoglyceric acid and 2-phosphoglyceric acid, but the yield from the latter compound was very low. According to the results, it is most probable that the chemically synthetic adenylyl 2,3-diphosphoglyceric acid has pyrophosphate bridge at the 3-phosphate of diphosphoglyceric acid rather than at 2-phosphate. The natural nucleotide will be the same AMP-3-phospho anhydride because of the identical properties with the synthetic nucleotide in several analysis.

We thank Dr. H. Hayatsu for his advice to call our attention to the use of adenosine-5'-phosphoromorpholidate.

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Research on Pancreatic Ribonuclease

Property of Lyxo-uridine 2', 3'-cyclic Phosphate as a Substrate of Ribonuclease-IA

In the previous paper (*1*), the present authors reported the comparative activity of various substrate analogues in their competitive inhibition to the enzymatic action of pancreatic ribonuclease-IA on uridine or cytidine-2',3'-cyclic phosphate, and pointed out that the active site of the enzyme should be composed of at least three regions comprising the functional groups each of which responds to one of the three partial structures, *i. e.* base, sugar and phosphoryl groups of the substrate. Thus, it is conceivable that, among these three resions the two which interact with base and phosphoryl residues in the substrate molecule should involve the proton-attracting anionic (*2*) and proton-donating basic (*3*) groups respectively, and the third region should also, to some extent, interact with the sugar moiety of the substrate.

As these three regions are considered to be distributed in the three dimensional structure of the enzyme protein, the substrate molecule, which will be held on the active site, is required to have a properly configurated structure.

In order to have more precise picture in this problem, the present authors synthesized lyxo-uridine 2',3'-cyclic phosphate (I) (*4*), which has an entirely similar structure to that of a natural substrate, uridine 2',3'-cyclic phosphate (II), except the configurations at C-2' and C-3', that is, the cyclic phosphodiester group of (I) is located in the opposite side of the pentafuranose ring to that of (II).

This stereo-isomer of (II) was tested as for its property as a possible substrate of RNase-IA. As is given in Table I, even with increased amount of the enzyme, no evidence of hydrolysis of (I) to the corresponding phosphomonoester, lyxo-uridine 3'-phosphate, was observed. Table II shows the inhibitory activity of lyxo-uridine 2'(+3')-(III) (*4*) and 5'-phosphates (IV) (*4*) as well as (I) on the hydrolysis of cytidine 2',3'-cyclic phosphate by RNase-IA. The inhibition indices of (III) and (IV) were very small compared with that of uridine 2'(+3')-phosphate and were close to that found for

TABLE I

Hydrolysis of Cyclic Phosphates by RNase-IA[a]

Substrate	Substrate/Enzyme	Per cent of hydrolysis		
		0.5hr.	7hr.	54hr.
(i) Lyxo-uridine 2',3'-cyclic phosphate[b]	10/5	0	0	0
(ii) Lyxo-uridine 2',3'-cyclic phosphate[b]	10/50	0	0	0
(iii) Uridine 2',3'-cyclic phosphate (as a comparison)	10/5	100	100	—

a) RNase-IA was prepared according to the method of Kunitz (*5*) and Hirs *et al.* (*6*).

The solutions of 1 mg. of the ammonium salt of the cyclic phosphate and each 0.5 mg. (i) (iii) and 5 mg. (ii) of RNase-IA in 0.2 ml. of *M*/10 Tris buffer (pH 7.5) were incubated at 37–39°C. The reaction was pursuedby paper chromatography (solvent: *iso*PrOH-conc. NH_4OH-H_2O, 7–1–2). Blank solutions were prepared similarly as above without the addition of RNase-IA and incubated at the same time with the test solutions. The blanks exhibited no hydrolysis within the assay period.

b) Ammonium salt of lyxo-uridine 2',3'-cyclic phosphate was prepared as follows: an aqueous solution of barium lyxo-uridine 2',3'-cyclic phosphate (*4*) was passed through a column of Dowex 50 (ammonium form) resin and the effluent was lyophilized to give chromatographically pure ammonium salt.

inorganic phosphate (*1*). The cyclic phosphate (I), on the other hand, showed practically no inhibition.

TABLE II

Inhibition by Several Lyxouridine Phosphates on the Hydrolysis of Cytidine2,3'-cyclic Phosphate by RNase-IA[a)]

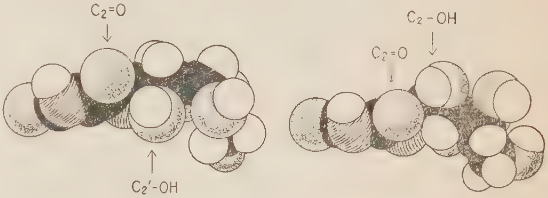
Inhibitor	Inhibition Index [b)]
Lyxo-uridine 2',3'-cyclic phosphate	6
Lyxo-uridine 2'(+3')—phosphate	22
Lyxo-uridine 5'-phosphate	22
Uridine 2'(+3')-phosphate (as a comparison)	73

a) As has been reported in the preceding paper (*1*), 0.5 ml. of enzyme solution containing 40 μg. of RNase-IA was placed into the side arm of Warburg manometer. 1.0 ml. of an aqueous solution containing 30.8 μmoles of ammonium cytidine 2',3'-cyclic phosphate, 1.0 ml. of 0.1 *N* sodium bicarbonate solution and 1.0 ml. of inhibitor-compound solution (containing equimolar ammonium salt to the substrate used) were introduced successively into the main compartment of flasks and the system was throughly equilibrated with a mixture of nitrogen and carbon dioxide (95 : 5 . The experiment was performed at 37°C and pH of the reaction mixture was 7.6. The control system contained a sodium chloride solution of the same ionic strength as the test solution. Readings of carbon dioxide evolution were made every three minutes and plotted against time to give linear slopes.

b) The decrease of the tangent of a slope from that of the control: represented by per cent (*1*).

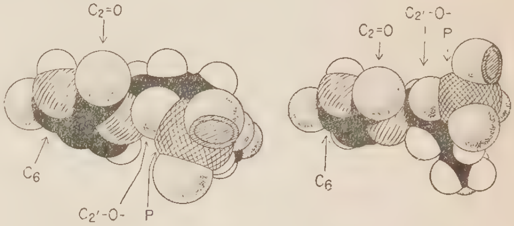
These results reveal that lyxo-uridine 2', 3'-cyclic phosphate and its hydrolysis product (III) can not be held on the active site of the enzyme.

It is interesting that as is illustrated in Fig. 1a, in the molecule of lyxo-uridine, the hydroxyl group at C-2' position, similarly to the case of uridine or cytidine, is sterically well situated to be able to interact with the carbonyl group at C-2 position which is activated by mesomeric effect in pyrimidine ring (*3*). The observed bathochromic shift of the ultraviolet absorption maximum of lyxo-uridine with increase of pH (λ_{max}^{pH12} 261 mμ (ε, 7430), λ_{max}^{pH13} 262.5 (7,650), λ_{max}^{pH14} 264 (7,950)) also indicated the interaction between these two groups (*7*). Thus, lyxo-uridine 2',3'-cyclic phosphate can, in this respect, fulfil the structural requirement which was postulated by Witzel (*3*) for the active substrate of RNase-IA.



Lyxo-uridine uridine

FIG. 1a. Molecular models of lyxo-uridine and uridine.



Lixo-uridine 2'3'-cyclic phosphate uridine 2' 3'-cyclic phosphate

FIG. 1b. Molecular models of lyxo-uridine 2',3'-cyclic phosphate and (I) uridine 2',3'-cyclic phosphate. (II)

The failure of the compound (I) to serve as a substrate of the enzyme must, consequently, be due to its difference from (II) in the relative location of phosphoryl moiety to the pyrimidine ring. The molecular models of (I) and (II) (Fig. 1b) show that the distance between phosphorus and C-6 of the pyrimidine ring in (I) and (II) is ca. 6.6 Å and 7.8Å respectively.

As the result of above discussions, not only the intramolecular activation of C-2' O-P bond by C-2 carbonyl group but also the proper locations of the phosphoryl and pyrimidine residues in the molecule should be an important factor for a pyrimidine cyclic nucleotide to form Michaelis' complex with RNase-IA and to be hydrolyzed by this enzyme.

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A Sulfite Reductase from *Aspergills nidulans*

Cell-free extracts from yeast and *Escherichia coli* have been reported to catalyse the reduction of sulfite using TPNH as hydrogen donor (*1–3*). Ishimoto *et al.* (*4*), on the other hand, have shown that reduced methylviologen (MVH) acts as an effective donor for the reduction of sulfite to sulfide by an enzyme preparation from *Desulfovibrio desulfuricans.* More recently, Ishimoto and Fujimoto (*5*) have further reported that an active MVH-sulfite reductase is also present in extracts from *E. coli.* In the course of a study on the pathway of sulfate assimilation in *Aspergillus nidulans,* we have also noted that this mold contains a similar MVH-sulfite reductase. Neither DPNH nor TPNH was, however, effective as hydrogen donor.

A. nidulans was cultivated for 50 hours in the "sulfate medium" as described previously (*6*). The mycelia were washed, ground with quartz sand, and extracted with 0.05 *M* Tris buffer, pH 7.5. The extract was centrifuged at 10,000×*g* for 15 minutes to remove debris and quartz sand. The crude extract thus obtained was dialysed overnight against 0.05 *M* Tris buffer, pH 7.5.

Despite various attempts, no reduction of sulfite could be detected with both dialysed and undialysed extracts using DPNH or TPNH as hydrogen donor. It was, however, readily demonstrable that the extracts contained a MVH-sulfite reductase activity. As is illustrated in Fig. 1, the addition of sulfite to an anaerobic mixture of MVH and the extract caused a rapid oxidation of the reduced dye. The dialysed extract was more suitable for this type of experiments, since undialysed extracts contained endogenous substances which oxidized MVH even in the absence of added sulfite. The enzyme activity was completely destroyed by heating the extract at 100°C for 5 minutes, but was stable for at least two months when stored at −15°C. The enzyme was most active at pH 7.5 and no dialysable cofactors seemed to be required for its activity. It appears to be localized in the soluble fraction of mycelia, since all of the activity was recovered in the supernatant when the dialysed extract was centrifuged at 100,000×*g* for 60 minutes. It was also possible to purify the enzyme about 100-fold by ammonium sulfate fractionation followed by column chromatography on diethylaminoethyl cellulose.

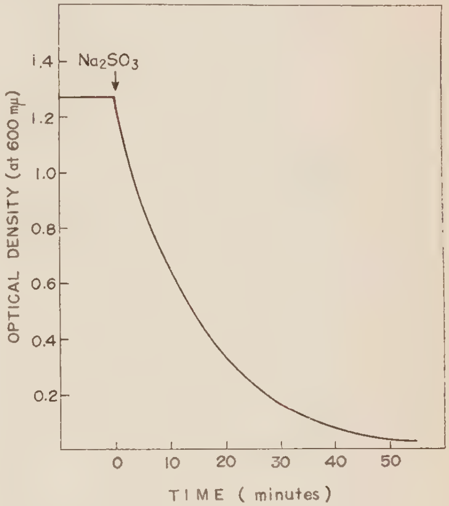

FIG. 1. Spectrophotometric demonstration of sulfite reductase. The reaction was carried out anaerobically in a Thunberg-type cuvette at 20°C. The reaction mixture contained 150 μmoles Tris buffer, pH 7.5, dialysed fungal extract (0.94 mg. protein), and MVH (prepared by reducing MV with H_2 and a Pd catalyst and anaerobically transferred into the cuvette) in a total volume of 2.9 ml. After preincubation for 50 minutes, the reaction was started by adding 3 μmoles Na_2SO_3 (0.1 ml.) from the side arm and the optical density at 600 mμ was followed in a Cary model 14 recording spectrophotometer.

Ishimoto and Fujimoto (*5*) manometrically measured the activity of *E. coli* sulfite reductase by coupling it with hydrogenase. Since this method of assay seemed more convenient for quantitative studies, similar experiments were performed also with the dialysed extract from *A. nidulans.* The hydrogenase used for this purpose was prepared from *D. desulfuricans* according to Ishimoto and Koyama (*7*) and shown to be free from both sulfite and thiosulfate reductases. When sulfite was added to a reaction mixture containing the hydrogenase preparation, methylviologen (MV) and *A. nidulans* extract in a hydrogen atmosphere, active consumption of hydrogen was actually observed indicating the operation of the following hydrogen transfer system:

TABLE I

Stoichiometry of Sulfite Reduction

Na_2SO_3 added (μmoles)	Hydrogen consumed (μmoles)	Sulfide formed (μmoles)
0	0	0.10
1.00	2.76	0.97
2.00	5.88	1.66

The main compartment of manometric vessel contained 300 μmoles Tris buffer, pH 7.5, 3 mg. bovine serum albumin, 6 μmoles MV, 200 units *D. desulfuricans* hydrogenase, and dialysed fungal extract (2.33 mg. protein) in a total volume of 2.8 ml. Gas phase, hydrogen. Temperature, 30°C. The center well contained 0.2 ml. 20 per cent KOH and a small strip of filter paper. The reaction was started by tipping 0.2 ml. Na_2SO_3 from the first side arm. After 200 minutes, 0.4 ml. of 3 N H_2SO_4 was added from the second side arm and shaken for 30 minutes to allow the evolved H_2S to be completely absorbed in KOH which was then analysed for sulfide.

$$H_2 \rightarrow \text{hydrogenase} \rightarrow \text{MV} \rightarrow \text{sulfite reductase} \rightarrow SO_3^{--}$$

No hydrogen uptake took place in control experiments without sulfite or with boiled fungal extract. In these experiments an excess of hydrogenase was added so as to make sure that the overall rate of reaction was limited by the sulfite reductase activity in the fungal extract.

After the hydrogen uptake had come to standstill, the reaction was stopped and the mixtures were analysed for sulfide. The amounts of sulfide thus determined are recorded in Table I together with those of sulfite added and hydrogen consumed. It will be clear from these data that the sulfite added was completely reduced to sulfide according to the following equation:

$$SO_3^{--} + 3H_2 \rightarrow S^{--} + 3H_2O$$

As MV is entirely foreign to living systems, it is expected that a natural substance is acting as hydrogen donor *in vivo* for this enzyme. The identification of such a natural donor is to be explored in the future.

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